University of Alberta

Apolipoprotein-B48 in Overweight Pre-Pubertal Children

By

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©

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Abstract

Cardiovascular disease (CVD), caused mainly by atherosclerosis, is the primary cause of death worldwide. Atherosclerosis has its genesis early in life, thus childhood is the ideal period for prevention and intervention. Novel lipid subfractions, such as intestinally-derived chylomicrons, have been shown to play a pivotal role in the initiation and progression of atherosclerosis. Since the prevalence of other known risk factors for CVD (e.g. obesity) is increasing in children, there is an urgent need to characterize chylomicrons in the pediatric population. The purpose of this study was to determine whether apolipoprotein-B48 (apo-B48) associated chylomicrons contributed to the CVD risk profile in overweight pre-pubertal children. We demonstrated that apo-B48 concentration was elevated in this group compared to published reports in adult populations. However, apo-B48 did not cluster with other known risk factors for CVD (e.g. pre-diabetes, hypertension and adiposity). Further studies are needed to delineate our findings.

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List of Abbreviations

Apo-B48	Apolipoprotein-B48
ASP	Acylation-Stimulation Protein
BF	Body Fat
CAD	Coronary Artery Disease
CAM	Cell Adhesion Molecule
CDC	Centers for Disease Control
CIU	Clinical Investigations Unit
CI	Confidence Interval
CN	Catalogue Number
CR	Chylomicron Remnant
CVD	Cardiovascular Disease
D	Density
DBP	Diastolic Blood Pressure
ECAM-1	Endothelial Cellular Adhesion Molecule-1
ECL	Electrochemiluminescence
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FISP	Fast Imaging with Steady Precession
FoV	Field of View
GAG	Glycosaminoglycans
HASTE	Half-Fourier Acquisition Single Shot Turbo Spin Echo
HASTE water SAT	HASTE water saturation
HDL	High Density Lipoprotein
HNRU	Human Nutrition Research Unit
HOMA	Homeostasis Model Assessment of Insulin Resistance
IDL	Intermediate Density Lipoprotein
IGT	Impaired Glucose Tolerance
IQR	Interquartile Range
IMT	Intima Media Thickness
LDL-C	Low Density Lipoprotein Cholesterol
MRI	Magnetic Resonance Imaging
NCEP-ATPIII	National Cholesterol Education Program Adult Treatment Panel III
NO	Nitric Oxide
OR	Odds Ratio
PAF-AH	Platelet Activating Factor Acetylhydrolase
PAI-1	Plasminogen Activator Inhibitor-1
RE	Retinyl Esters
RLP	Remnant Lipoprotein
SAT	Subcutaneous Abdominal Adipose Tissue
SBP	Systolic Blood Pressure
SD	Standard Deviation
T2DM	Type 2 Diabetes Mellitus
TA	Acquisition Time

TC	Total Cholesterol
TFM	Trunk Fat Mass
TE	Echo Time
TG	Triglyceride
TLM	Trunk Lean Mass
TR	Repetition Time
TRL	Triglyceride Rich Lipoprotein
TSAAT	Total Subcutaneous Abdominal Adipose Tissue
TVAT	Total Visceral Adipose Tissue
UAH	University of Alberta Hospital
USAAT	Umbilical Subcutaneous Abdominal Adipose Tissue
UVAT	Umbilical Visceral Adipose Tissue
VCAM-1	Vascular Adhesion Molecule-1

Chapter 1: Introduction

1.1 Rationale

Arguably our nation is facing one of its greatest public health crises: Canadian children are getting heavier at an alarming rate. The prevalence of pediatric obesity[1] has increased dramatically in recent years. Twenty five years ago, 1% of children had a body mass index (BMI) $\geq 95^{th}$ percentile. Today, 8% of 6 to 11 year olds have a BMI above this threshold. Originally dismissed as a cosmetic issue, it is now clear that the consequences of childhood obesity extend far beyond aesthetics. Excess weight in childhood increases a person's risk of morbidity and premature mortality, especially due to cardiovascular disease (CVD).

The number one cause of death in Canada is CVD. In 2003, CVD alone killed 73,827 Canadians. Cardiovascular disease accounts for $19.7 billion in direct and indirect cost of illness and continues to be the single greatest disease category expenditure in Canada (Heart and Stroke Foundation of Canada, 2000). Early diagnosis and treatment of CVD are essential to reduce its substantial medical, economic and social burdens. Coronary artery disease (CAD) and cerebrovascular disease are the most common cardiovascular diseases; both are caused by atherosclerosis, a chronic condition that has its genesis early in life.

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[1] The terms *overweight* and *obese* are used with varying definitions in the literature. For the purpose of this thesis, these terms will be used interchangeably and refer to children with a high level of body fat. When discussing other studies, the terms used in the papers will be used and definitions will be provided when necessary.

Atherosclerosis mainly affects the coronary and aortic vessels. While end-stage atherosclerosis is associated with older adulthood, the disease process is initiated in early childhood (McGill *et al.*, 1963). Atherosclerosis begins with lipid deposition in the subendothelial space and a subsequent inflammatory response (Gustafsson *et al.*, 2004). Acutely, this inflammatory response may be beneficial as it metabolizes lipid particles in the vessel wall (Libby *et al.*, 2002). However, chronic lipid accumulation induces a maladaptive response that is thought to ultimately lead to atheroma and occlusion of the vessel. The etiology of atherosclerosis is complex and despite years of research, not completely understood. Further, initiation of atherosclerosis and its progression are difficult to study in humans.

The complexity of studying atherosclerosis in humans is further increased by the influence of gender on CVD morbidity and mortality. Until menopause, women have a substantially lower risk of CVD compared to men (Thom *et al.*, 2006). Based on this evidence, it is thought that sex hormones influence the progression of atherosclerosis. Current pediatric data are equivocal with regards to the influence of gender on risk of CVD and further study, particularly in the pre-pubertal population, is important to understand the influence of gender on CVD risk. Today, risk factors and clinical events (i.e., heart attack or stroke) are used clinically to estimate disease risk, progression and severity.

Traditionally, clinicians have relied on measuring cholesterol (particularly low-density lipoprotein cholesterol (LDL-C)) as a key indicator of CVD risk in adults (NCEP, 2002). However, many patients who present with a clinical event have normal LDL-C, suggesting that other CVD risk factors play important roles (Mamo & Proctor, 2002). The challenge to identify those at risk of developing CVD is greater in children because LDL-C is generally normal, even among high risk individuals (e.g., overweight, insulin resistant). Thus, there remains an urgent need to identify novel markers in children to facilitate early identification of CVD risk.

Post-prandial lipids are an important component in the development of atherosclerosis (Karpe *et al.,* 1994). Closer examination of specific lipid sub-fractions has led to the identification of intestinally-derived chylomicrons as important contributors to CVD development. Chylomicrons are associated with the initiation and progression of atherosclerosis because small triglyceride-rich remnant particles, like chylomicron remnants (CRs), can penetrate the vessel wall and become entrapped in the sub-endothelial space (Simionescu & Simionescu, 1991b, Hurt-Camejo *et al.*, 1997). Chylomicrons carry cholesterol from the intestine to the circulation after ingestion of a fatty meal (Redgrave, 2004). Our laboratory has previously shown that chylomicrons can deliver more cholesterol to the vessel wall than the LDL-C lipid subfraction (Proctor *et al.*, 2002). In disease states, production and secretion of chylomicron particles have been shown to be up-regulated (Chen *et al.*, 1993, Karpe *et al.*, 1994, Hirany *et* *al.*, 2000). Furthermore, in the context of insulin resistance and obesity, chylomicron concentrations are elevated due to poor hydrolysis and hepatic-mediated clearance impairment (Mamo *et al.,* 2001). It is known that elevated chylomicron concentrations cluster with other known risk factors for CVD including insulin resistance, hypertriglyceridemia and visceral obesity (Mamo *et al.,* 2001). However, it is unknown if chylomicrons are similarly associated with risk factors for CVD in children.

To our knowledge, no published reports have examined chylomicrons in pre-pubertal, overweight children. Further, the propensity of elevated chylomicron concentrations to cluster with other known risk factors for CVD has not been evaluated in the pediatric population. Therefore, the assessment of chylomicron concentration as a novel marker of CVD risk is the focal point of this thesis.

1.2 Thesis Aim

The overall aim of this study was to determine whether plasma apo-B48 associated-chylomicron concentrations contributed to the CVD risk profile in an overweight, pre-pubertal, pediatric population.

1.3 Specific Hypothesis

The hypotheses of this thesis were as follows:

1. Plasma apolipoprotein B-48 (apo-B48) associated-chylomicron concentration is elevated in overweight, pre-pubertal children.
2. Elevated apo-B48 will correlate and / or cluster with established CVD risk factors including a dyslipidemic profile (elevated total cholesterol [TC], elevated triglycerides [TG], reduced high density lipoprotein cholesterol [HDL-C] and elevated LDL-C), pre-diabetes (insulin resistance [IR] and impaired glucose tolerance [IGT]), high blood pressure (BP), and measures of adiposity (e.g., body mass index (BMI), and percent body fat (%BF).
3. Apo-B48 concentration and its relationship to the dyslipidemic profile, pre-diabetes, blood pressure and adiposity will not differ between boys and girls.

1.4 Specific Objectives

To test the above hypotheses, we sought:

1. To establish fasting plasma apo-B48 concentrations in overweight, pre-pubertal children and compare to published adult data.
2. To examine the relationship between apo-B48 and other lipid parameters, pre-diabetes, blood pressure and adiposity and to characterize the contribution of apo-B48 to risk factor clustering by assigning participants to a high risk or moderate risk group (according to defined cut-off points for dyslipidemia, IR, IGT, elevated blood pressure, BMI and %BF) and compare apo-B48 concentrations between groups.
3. To examine the influence of gender on apo-B48 and its relationship to other lipid parameters, pre-diabetes, blood pressure and adiposity.

Chapter 2: Literature review

2.1 Overweight and obesity[2]

2.1.1 Introduction: Health Canada (2006) recently identified obesity as a critical Canadian public health challenge. Unequivocally, obesity is a major, modifiable risk factor for CVD (Must *et al.,* 1999; Woo *et al.,* 2004) that also increases an individual's risk for hypertension, type 2 diabetes mellitus (T2DM), gallbladder disease, osteoarthritis, sleep apnea, colon cancer, endometrial cancer and mental health problems like low-self esteem and depression (Health Canada, 2006). Since 1981, adult obesity (20-64 year olds, BMI ≥ 30 kg/m^2) has increased from 9 to 14% in men and 8 to 12% in women (Tremblay *et al.,* 2002). The expansion in adult obesity is modest in comparison to the 5-fold (from 2% to 10%) increase in overweight (BMI > 95th percentile) in the past 25 years among Canadian children between the ages of 7 and 13 (Shields, 2006). Pediatric obesity has become a particular concern for public health institutions, clinicians and researchers for three major reasons (Goran *et al.,* 2003): (1) the prevalence of pediatric obesity is high and on the rise; (2) obesity in childhood persists into adulthood; and (3) obesity increases risk of morbidity and premature mortality. In order to reinforce the rationale for our study, it is important to expand upon these three points.

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[2] The terms *overweight* and *obese* are used with varying definitions in the literature. For the purpose of this thesis, these terms will be used interchangeably and refer to children with a high level of body fat. When discussing other studies, the terms used in the papers will be used and definitions will be provided when necessary.

2.1.2 Obesity prevalence estimates in pre-pubertal children: Three national databases, including the 1981 Canada Fitness Survey (7-19 years old; number (n) = 4176), the 1988 Campbell's Survey on the Well-being of Canadians (7-19 years old; n = 481), and the 1996 Canadian National Longitudinal Survey of Children and Youth (0-13 years old; n = 7847), were used to ascertain the change in the prevalence of overweight among Canadian children between 1981 and 1996. The prevalence of overweight (≥95th percentile) increased from 2% to 10% in boys and from 2% to 9% in girls, aged 7-10 years old (Tremblay *et al.*, 2002). Over the past 25 years in Canada, the prevalence of overweight has increased to a greater degree among children (6-11 years old; 1 to 8%) compared to adolescents (12-17 years old; 3% to 9%) (Shields, 2006). As such, it is thought that the pre-pubertal period is an important period for weight gain. A state-wide American study found that especially in girls (n = 2379), the risk of becoming overweight in childhood was 1.7 times (confidence interval (CI): 1.2-2.5) greater between the ages of 9 and 12 than above 12 years old (Thompson *et al.,* 2007). Although, to our knowledge, a similar study in boys has not yet been conducted, these data suggest that the pre-pubertal stage may be a key period for risk evaluation and intervention.

2.1.3 Persistence of childhood overweight into adulthood: Ferraro *et al.* (2003) conducted a 20-year, retrospective analysis using data from the National Health and Nutritional Examination Survey 1: Epidemiological Follow-up Study to examine the relationship between severe obesity (BMI $\geq$35 kg/m^2) in childhood (self-reported) and adulthood (measured). Severely obese individuals at 12 or 13 years of age (n = 338) were 5.2 times (CI: 4.0-6.9) more likely to be severely obese in adulthood compared to those who were not obese in childhood (n = 6435). Further analysis indicated that the strongest predictor of long-term obesity was the presence of overweight in childhood. This trend was similar in men and women, however childhood obesity had a greater effect on severe adult obesity in men (n = 3105) compared to women (n = 3623) (odds ratio (OR) = 8.6 *versus* 4.2; respectively). A prospective study of 103 Italian children (Maffeis *et al.*, 2002) found that 43% of the boys and girls who were obese (BMI $\geq$ 95th percentile) in childhood (8 -13 years old; n = 79) remained obese (BMI > 30 kg/m^2) in adulthood (17 - 31 years old; n = 34). In this study, childhood overweight was the strongest predictor of adult obesity, but this association was only significant in girls. An examination of 2617 participants from the Bogalusa Heart Study (Freedman *et al.*, 2001), who were followed for approximately 17 years, revealed that adults who had been overweight as children (n = 342) had an adult BMI 12.4 kg/m^2 higher than adults who were normal weight as children (n = 2363) (34.9 kg/m^2 *versus* 22.5 kg/m^2; respectively). Further, 77% of overweight (BMI $\geq$95th percentile) children (n = 186) remained obese (BMI $\geq$30 kg/m^2; n = 144) as adults compared to only 7% of non-overweight children (n = 1317) who became obese as adults (n = 96). Gender did not significantly influence these findings. Collectively, the data suggest that excess weight in childhood is linked to an increased BMI in adulthood. There may be some differences in the relative importance of childhood obesity to adult obesity in men compared to women; however, the influence of gender is equivocal. In addition, the epidemiological link between adult obesity and disease risk suggests that childhood obesity is potentially predictive of adult morbidity and mortality.

2.1.4 Pediatric obesity and risk factors for chronic diseases: Pediatric obesity is associated with an increased prevalence of dyslipidemia, hypertension, IR, polycystic ovarian syndrome, hepatic steatosis, orthopedic concerns, psychosocial issues, sleep apnea, and asthma (Bell *et al.*, 2007; Deckelbaum & Williams, 2001; Young-Hyman *et al.,* 2006). As well, evidence suggests that childhood overweight increases an individual's risk of CVD mortality in adulthood. For example, overweight children may be at increased risk of premature mortality, particularly from CAD (relative risk (RR) = 1.7-2.5) (Freedman, 2002). In a 57 year follow up study in British men and women (Gunnell *et al.*, 1998), the overweight group (BMI $\geq 90^{th}$ percentile; n = 192/2399) was 2.82 times (95^{th} percentile CI: 0.74-2.31) more likely to die of ischemic heart disease compared to the normal weight group (25^{th} percentile < BMI < 49^{th} percentile). In addition, the hazard ratio (RR) for all cause mortality was 1.31 (CI: 1.26-6.33) times greater among the overweight group compared to the normal weight group. Although women exhibited a lower mortality rate (2.8 *versus* 4.2 per 1000 people, respectively), gender was not a significant covariate in the relationship between BMI and CVD mortality. Also, research indicates that the degree of risk for some factors increases with weight severity. For instance, Bell *et al.* (2007)

demonstrated that HDL-C decreased and TG increased exponentially with BMI Z-scores between 0 and 3, among 177 children (6-13 year old).

2.1.5 Conclusion: Conducting our study in the pre-pubertal, overweight pediatric population is essential. Since evidence suggests that (1) pre-puberty is a key period for excess weight gain (Thompson *et al.*, 2007), (2) the prevalence of overweight in the pre-pubertal Canadian population has increased dramatically in the past 25 years, (3) childhood obesity is strongly related to adult weight, (4) the pre-pubertal period may be a key age group to examine risk of CVD with minimal gender influences, since within this age group gender does not alter the important link between obesity and risk of CVD, (4) obesity and its related health risks (particularly IR, dyslipidemia and hypertension) put children at increased risk for developing CVD in adulthood (Freedman, 2002), we predict that within the pediatric population, pre-pubertal, overweight children are likely at greatest risk of developing CVD-related risk factors and thus an important age group to study in order to further elucidate the CVD-risk factor profile.

2.2 Cardiovascular disease

2.2.1 Introduction: Cardiovascular disease is the primary cause of death worldwide (WHO, 2003). In 2005, 17.5 million people died of CVD; 7.6 million from CAD and 5.7 million from stroke (WHO, 2007). The 1999 Heart and Stroke Foundation report revealed that CVD was responsible for 36% of Canadian deaths. Specifically, 20.2% of deaths were attributed to heart attacks and 10% to stroke. In 2003, 33% of people (36,823 women and 37,004 men) died of heart attacks and stroke (Heart and Stroke Foundation, 2007). Despite the decrease in CVD-related mortality between 1997 and 2003, the Heart and Stroke Foundation predicted that in the next ten years, there would be 100,000 more hospitalizations per year due to CVD (Heart and Stroke Foundation, 2007). Cardiovascular disease also confers a substantial economic burden. The most recent economic analysis reported that CVD accounted for $7.3 billion annually or 17% in direct cost of illness (hospital care, medical expenditure, drugs and research) and $12.3 billion annually or 14.5% of indirect costs (loss of productivity due to illness or disability and loss of future earnings due to premature death) (Public Health Agency of Canada, 2002).

Cardiovascular disease, by definition, includes a number of conditions involving the heart and its vessels. Cerebrovascular disease, CAD, peripheral artery disease, rheumatic arterial disease, congenital heart disease, deep vein thrombosis and pulmonary embolism are all included under the umbrella term

"CVD"[4] (WHO, 2007). The two most common disorders are CAD (blockage or occlusion of the coronary vessels) and cerebrovascular disease (blockage or occlusion of the cerebrovasculature) (WHO, 2007). Occlusion of arteries that supply the heart or brain, as seen in advanced CAD or cerebrovascular disease, results in a heart attack or stroke. Vessels become occluded by fatty lesions leading to atheroma, a build up of lipid, T-lymphocytes, foam cells, extracellular matrix and fibrous tissue. Development of these fatty lesions initiates progressive and chronic atherosclerosis (Berenson *et al.,* 1998).

It is well established that gender substantially influences the prevalence and mortality of CVD in adults. Despite an overall reduction in mortality attributable to CAD (Heart and Stroke Foundation, 2007), compared to women, men have a greater prevalence of CAD, but only until women reach menopause; post-menopause, the prevalence of CAD in women exceeds that of men (Thom *et al.*, 2006). Based on this evidence, a hypothesis that estrogens are cardioprotective in women was proposed (**Table 2-1**). Epidemiological evidence has largely supported this theory. For instance, when total lifetime exposure to endogenous estrogen was calculated (based on the age of menarche to the age of menopause), it was found that women with longer total lifetime exposure had a decreased number of heart attacks (Saltiki *et al.,* 2006). Using coronary angiography, Bairey *et al.* (2003) found pre-menopausal women with lower estrogen levels tended to have more severe atherosclerosis compared to their counterparts with higher estrogen levels. Recent research indicates that testosterone can be similarly cardioprotective in men; however, there is a progressive decrease in testosterone with age which plays a role in the increased prevalence of CVD in men (English *et al.*, 2000) (**Table 2-1**).

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[4] Although CVD includes many conditions, studies cited in this thesis refer only to CVD leading to heart attack, ischemic heart disease and stroke (i.e., CAD and cerebrovascular disease).

Table 2-1 Favourable effects of estrogens and androgens on vasculature and metabolism relating to CVD

	Effects	**References**
Estrogens	Inhibits plaque calcification.	Hofbauer & Schoppet, 2001
	Inhibits proliferation of endothelial smooth muscle cells.	Mendelsohn & Karas, 1999
	Inhibits thickening of carotid intima medial layer.	Sutton-Tyrrell *et al.*, 1998
	Promotes vasodilation via promoting production of vasodilatory species (i.e., nitric oxide and prostacyclin).	Mendelsohn & Karas, 1999
	Inhibits production of vasoconstrictors (i.e., endothelin-1).	Medelsohn *et al.*, 2002
	Promotes the gynoid body fat partitioning pattern.	Klouche, 2006
	Promotes a favourable lipid profile (i.e., low LDL-C and high HDL-C).	Rosano *et al.*, 2007
	Decreases secretion of proinflammatory molecules, decreases vascular adhesion expression and leukocyte activity in the endothelium.	Saltiki *et al.*, 2006
	Increases glucose tolerance	Rosano *et al.*, 2007
Androgens	Inhibits thickening of endothelial intima medial layer.	Muller *et al.*, 2004
	Associated with lower BMI, increased lean body mass and decreased visceral adipose tissue	Rondinone, 2006
	Stimulates lipolysis.	Marin *et al.*, 1996
	Decreases (indirectly) circulating adipocytokines.	Rondinone, 2006
	Decreases insulin resistance.	Ding *et al.*, 2006

Free and bioavailable testosterone levels decrease in men as they age as a result of the progressive increase in sex hormone-binding globulin (Feldman *et al.*, 2002). Up to 20% of men aged 60-69 years have hypogonadism (Cunningham, 2006). It is possible that the higher prevalence of CVD in men compared to women (pre-menopause) is, in part, due to decreasing levels of endogenous testosterone (Choi & McLauglin, 2007). Many of the mechanisms responsible for sex differences are poorly understood, yet it is clear that gender plays an important role in CVD risk. In order to minimize differences attributable to sex hormones, our study population was limited to pre-pubertal boys and girls. Within this group, including pre-pubertal children allowed us to investigate the atherosclerotic process which begins before puberty and is more severe in overweight, insulin resistant, hypertensive and dyslipidemic individuals (Berenson *et al.*, 1998; Stary, 1989; Strong & McGill, 1962).

2.2.2 Atherosclerosis: Although it is known that the progression of atherosclerosis is influenced by metabolic abnormalities, the etiology is not completely understood. Atherosclerosis has several stages of development (**Figure 2-1**). The first stage is the fatty streak, an intracellular accumulation of lipid in endothelial smooth muscle cells and macrophages in the vessel intima (McGill *et al.,* 1963). Initially, intracellular lipid is metabolized by macrophages and removed from the subendothelial space. However, as lipid accumulation increases and overwhelms the capacity of the macrophages, they necrotize and form foam cells, progressing to the advanced fatty streak stage (stage two) (**Figure 2-1**). As lipid accumulation continues the endothelium and leukocyte response progresses leading to more severe stages of the disease particularly in the second and third decades of life. In the third stage fatty streaks evolve into fibrous plaques (where the plaque is covered by a cap composed of extracellular matrix, platelets and collagen). The fourth stage involves the formation of potentially occluding plaques which can result in a cardiovascular event (McGill, 1963). As early as 1958, there has been published evidence of atherosclerotic lesions in children (Holman *et al.*, 1958; Strong *et al.*, 1958). In fact, fatty streaks in the coronary arteries and aorta have been reported in children as young as two (McGill *et al.*, 1963; Strong & McGill, 1962). However, most atherosclerotic lesions do not result in a clinical event and diagnosis of the severity of atherosclerotic lesions prior to a clinical event is challenging. Identifying clinically relevant risk factors in children has proven equally challenging. The progression of atherosclerosis is influenced by a number of factors including IR, elevated blood pressure, dyslipidemia and excess weight and since these risk factors tend to "cluster", they have been used to assess risk of CVD in children.

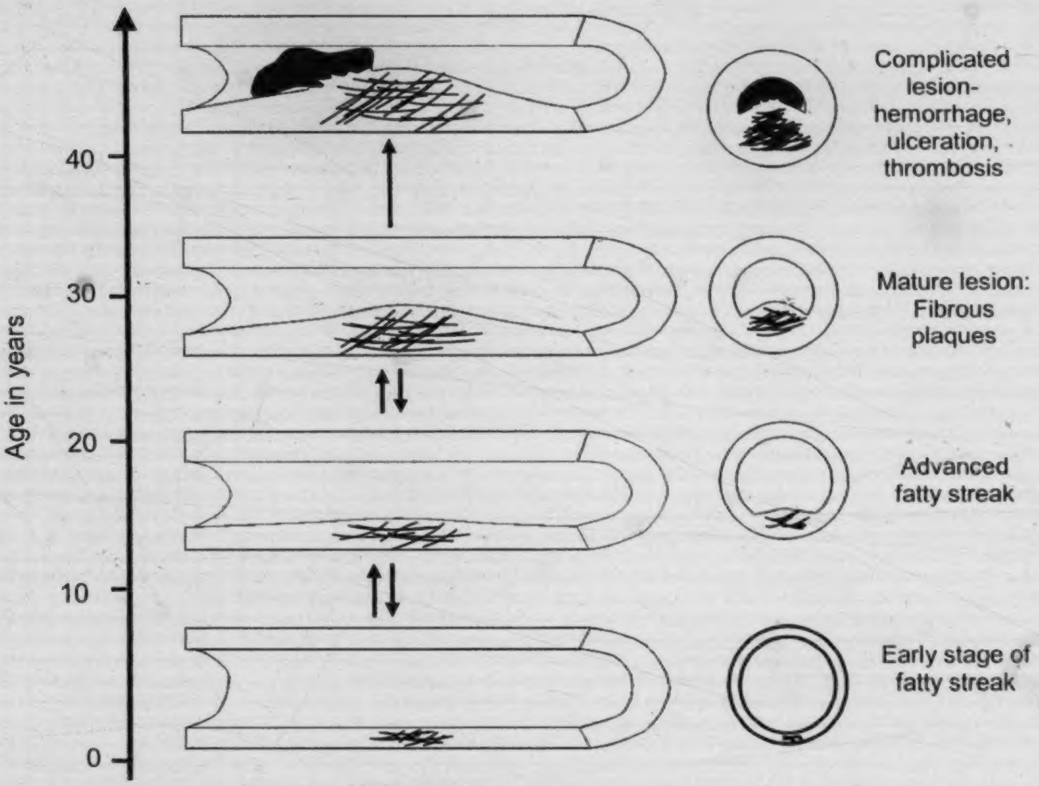
Age in years
0
10
20
30
40
Complicated lesion- hemorrhage, ulceration, thrombosis
Mature lesion: Fibrous plaques
Advanced fatty streak
Early stage of fatty streak

Figure 2-1 Diagram of the progression of atherosclerosis from childhood to adulthood. Adapted and redrawn from McGill *et al.*, 1963.

2.2.3 Risk factor clustering in children: Studies indicate that obesity, IR, dyslipidemia and hypertension confer additive risk for CVD. However, the absolute importance of each individual risk factor for CVD is unknown. (Weiss & Caprio, 2005). As a result, risk assessment for CVD is often evaluated in clusters (Arslanian & Suprasongsin, 1996; Kohen-Avramoglu *et al.*, 2003; Weiss & Caprio, 2005). To date universal criteria for risk factor clustering in children has not been established (Huang *et al.*, 2007). Varying combinations of elevated LDL-C, TC, TG, low HDL-C, hypertension, markers of inflammation, IR, IGT and measures of adiposity (e.g., BMI, waist circumference) have all been included in risk factor clustering analysis in children (Weiss *et al.*, 2004) (**Table 2-2**).

However, most studies included at least one measure of dyslipidemia, pre-diabetes, hypertension and obesity in their studies.

Table 2-2 Risk factors used in evaluating CVD risk

Risk factor	Measure	Established definitions
Obesity	WC or BMI	WC ≥90th percentile BMI ≥95th percentile, CDC growth charts
Insulin resistance	HOMA	No childhood age-specific definitions
Impaired glucose tolerance	2-hr glucose (from oral glucose tolerance test)	Glucose ≥7.8 mmol/L, CDA
Dyslipidemia (TC, LDL-C, TG, HDL-C)	Fasting plasma or serum concentrations	Borderline and / or high cut-offs (see Table 3-1), NCEP
Hypertension	SBP and / or DBP	SBP or DBP ≥95th percentile for three consecutive measurements, NHLBI
Endothelial dysfunction	Flow mediated dilation in the brachial artery	No childhood age-specific definitions
Intimal medial thickness	Thickness of the intima in the internal and common carotid arteries.	No childhood age-specific definitions
Inflammation (CRP, IL-6, TNF-α)	Fasting plasma or serum concentrations	No childhood age-specific definitions

Abbreviations: WC (waist circumference), BMI (body mass index), TC (total cholesterol); TG (triglyceride); HDL-C (high density lipoprotein); LDL-C (low density lipoprotein), CRP (C-reactive protein), IL-6 (interleukin-6), TNF-α (tumour necrosis factor-alpha), CDC (Centre for Disease Control), NCEP (National Cholesterol Education Program), NHLBI (National Heart, Lung, Blood Institute)

For instance, a Hungarian study (Csabi *et al.*, 2000) reported obese children (n = 180) were 19.4 times (CI: 11.6-32.3) more likely to have one risk factor for CVD and 6.3 times (CI: 3.0-13.4) more likely to have more than one risk factor (risk factors included hyperinsulinemia, hypertension, IGT and dyslipidemia). There were no significant differences reported between boys and girls. Iannuzzi *et al.* (2004) observed in 147 children that obese Italian children (n = 100) had significantly ($p < 0.05$) higher CRP concentrations, TG, TC, glucose, insulin, homeostasis model assessment of insulin resistance (HOMA) scores and BP compared to non-obese controls (n = 47). This trend was significant in both boys and girls. A German study, demonstrated that lower HDL-C levels, higher insulin, CRP concentrations, higher systolic blood pressure (SBP) and higher diastolic blood pressure (DBP) clustered significantly ($p < 0.001$) in obese children ($n = 96$) compared to non-obese children ($n = 25$) (Reinehr *et al.*, 2004). A report from the National Health and Nutrition Examination Survey (NHANES) 1999-2000 survey demonstrated that CRP concentrations were most strongly associated with BMI, SBP and TG. The association between TG and CRP was stronger for girls ($r = 0.25$) than boys ($r = 0.16$) (Ford, 2003). Similarly, a Canadian study including 9-year olds ($n = 257$) demonstrated that children with CRP concentrations in the upper quartile for their age were 1.4, 1.7, and 2.3 times, respectively, more likely to have high SBP, TG and low HDL compared to children in the lowest quartile for CRP concentrations (Lambert *et al.*, 2003). Compared to boys, girls had higher CRP concentrations (0.4 *versus* 0.2 mg/L; $p < 0.05$), higher TG concentrations (0.8 *versus* 0.7 mmol/L; $p < 0.05$) and higher plasma insulin concentrations (35.4 *versus* 29.4 pmol/L; $p < 0.05$), but risk factor clustering was similar in both sexes. Cruz *et al.* (2004) studied 126 overweight (BMI $\geq 85^{th}$ percentile), 8-13 year old Hispanic children in which 90% of children had at least one component of the "metabolic syndrome" (waist circumference (WC) $\geq 90^{th}$ percentile, TG $\geq 90^{th}$ percentile, HDL $\leq 10^{th}$ percentile, hypertension and IGT). The percentage of children with 2, 3 and 4 or 5 components was 38%, 30% and 10%, respectively. Again no gender differences were observed. Similar findings were observed in a Freedman *et al.* (1999) study. In this iteration of the Bogalusa Heart Study (9167 children, aged 5-17), fasting TC, TG, LDL-C, HDL-

C, insulin and BP were used to create a risk factor profile. Overweight children in the 5 -10 year old age group (BMI ≥95th percentile; n = 1705), were 9.7 times more likely to have 2 risk factors and 43.5 times more likely to have 3 risk factors compared to non-overweight children. This association was not significantly influenced by gender. A key edition of the Bogalusa Heart Study (Berenson *et al.*, 1998) examined how risk factor clustering related to the severity of atherosclerotic lesions. The study is particularly important because it provides the risk factors for CVD with clinical relevance, albeit retrospectively. In 93 individuals (2 to 39 years old), mean percentage of intimal surface covered by lesions was dependent upon the number of risk factors present. For instance, in the aorta and coronary arteries, intimal surface area covered by lesions increased with the number of risk factors. Subjects with 0 (n = 52), 1 (n = 14), 2 (n = 14), 3 or 4 (n = 7) risk factors had 19.1%, 30.3%, 37.9% and 35.0% vessel surface area covered by lesions in the aorta, respectively, and 1.3%, 2.5%, 7.9% and 11.0% of intimal surface in the coronary, respectively. Thus, subjects with 3 or 4 risk factors had an 8.5 times greater degree of fatty streak lesions in the coronary arteries, compared to patients with no risk factors. Similarly, the percentage of intimal surface covered by fibrous plaques, in the coronary arteries, was 12 times greater for the patients with 3 or 4 risk factors. The degree of lesions was adjusted for gender differences. Thus far, it has been difficult to identify a common underlying feature of risk factor clustering in children. The data suggest that a number of components, particularly IR and abdominal obesity, contribute individually and cumulatively to increased risk for chronic disease (Weiss, 2004; Cruz & Goran, 2004; Goran *et al.,* 2003). The contributions of both obesity and IR have been frequently explored in the literature, but a clinically relevant dyslipidemic profile remains to be characterized in children. The absence of data presents a need for CVD-related lipid research in the pediatric population.

2.2.4 Dyslipidemia: In the last three decades, the incidence of dyslipidemia has risen in the pediatric population. This change may be linked to the concomitant rise in pediatric obesity as demonstrated by Morrison *et al.* (1999). His group (Morrison *et al.,* 1999) examined changes in the plasma lipid profile of children (mean age: 10.0 ± 0.3 years) between two phases (1973-1975; n = 299 and 1989-1990; n = 1456) of the National Heart, Lung and Blood Institute (NHLBI) Lipid Research Clinics Princeton Cohort Study. Mean TC concentrations (164.6 ± 1.6 mg/dL *versus* 170.5 ± 0.9 mg/dL; $p < 0.01$) and mean TG concentrations (62.7 ± 1.7 mg/dL *versus* 66.4 ± 0.9 mg/L; $p < 0.01$) increased significantly between the 1973 and 1990 groups. Furthermore, the prevalence of hypercholesterolemia increased significantly ($p < 0.01$) from 8% (n = 24) to 14.8% (n = 150). In contrast, HDL-C and LDL-C levels did not change over time. Within the 17 year time period, changes in mean lipid concentrations, among the children, paralleled a gain in mean BMI (16.9 kg/m^2 to 18.3 kg/m^2). When analyzed according to gender, the increase in TC was only significant in Caucasian females and the increase in TG was significant in Caucasian males.

In addition, Caucasian females had a higher mean TG concentration compared to their male counterparts.

Another report from the Bogalusa Heart Study (Freedman *et al.*, 1999) showed that overweight children (BMI ≥95th percentile; n = 990), ages 5-17 years old, were more likely to exceed NCEP cut-offs for all the lipid parameters compared to non-overweight children (n = 8177). For example, overweight children were 2.4 times (CI: 2.0-3.0) more likely to have high TC levels, 7.1 times (CI: 5.8-8.6) more likely to have high TG levels, 3.0 times (CI: 2.4-3.6) more likely to have higher LDL-C levels, and 3.4 times (CI: 2.8-4.2) more likely to have lower HDL-C. Gender was not a significant influence on the association between weight and lipid parameters. In addition, among 1004 overweight German children, (BMI ≥ 90th percentile) 27% had hypercholesterolemia, 26% had elevated LDL-C, 18% had low HDL-C and 20% had hypertriglyceridemia. These percentages were not significantly influenced by gender (Reinehr *et al.,* 2005). Although the clinical relevance of classic lipid subfractions (e.g., LDL-C and TG) is well studied in adults, it has not been well defined in children. Furthermore, recent evidence suggests other lipid subfractions have an important role in atherogenesis. Although a number of studies have examined the persistence of dyslipidemia and other CVD risk factors from adolescence to adulthood, only one group (investigators involved in the Bogalusa Heart Study) has evaluated CVD risk factor persistence from pre-puberty.

2.2.5 Persistence of CVD risk from childhood into adulthood: The Bogalusa Heart Study has been essential in evaluating the persistence of CVD lipid risk factors in pre-pubertal children into adulthood. Over 50% of adults (n = 793/1586) who had elevated TC, LDL-C, low HDL-C and 35% who had elevated TG (n = 833/1586), had a corresponding lipid abnormality in childhood (12 years earlier) (Webber *et al.*, 1991). Furthermore, in an 1163-person cohort followed for an average of 27 years and controlling for gender, Srinivasan *et al.* (2006) reported that 66.2% subjects who were in the top two quintiles for LDL-C as children remained in their respective categories as adults, suggesting that elevations in lipid concentration tend to track over time. After childhood lipid concentrations, the degree of change in BMI from childhood to adulthood was the next best predictor of adult lipid concentrations which indicates that obesity plays an important role in the persistence of CVD risk into adulthood.

2.2.6 Conclusion: Although evidence shows that the prevalence of CVD risk factors, particularly dyslipidemia, is high in children and tends to persist into adulthood, further study into the risk factor clustering criteria, the influence of gender, and the contribution of dyslipidemia to CVD risk are required to better predict and treat those at greatest risk. Given that the process of atherosclerosis begins in early childhood, it is critical that risk assessment occurs at this stage in order to prevent atherosclerosis. The addition of other measures to the risk factor profile may facilitate early identification and treatment of children at risk of developing CVD.

2.3 Chylomicrons

2.3.1 Introduction: Since the early 1960s, scientists have been aware that chylomicrons play a role in the transport of dietary fat. However, it was thought that chylomicrons could not be directly involved in atherogenesis because nascent chylomicrons were too large to penetrate the vessel wall (Nordestgaard & Tybjaerg-Hansen, 1992). Work by Mamo *et al.* and Proctor *et al.* (Mamo & Wheeler, 1994; Mamo *et al.*, 1998; Proctor *et al.*, 2000; Proctor *et al.*, 2002) established that chylomicrons in their remnant form (post-hydrolysis) were in fact small enough to penetrate the vessel wall and become entrapped in the subendothelial space. Further research into the function of chylomicrons in atherosclerosis in humans has helped to elucidate their role.

2.3.2 Chylomicrons and Apolipoprotein B48: Chylomicrons are lipoprotein particles secreted from the intestine and their key function is to transport dietary fat in the circulation. In size, native chylomicrons can be the largest lipoprotein particle (up to 1000 nm) and they are considered to be endogenous because they transport dietary fat and are produced by the intestine. At their core is a complex of TG and cholesterol esters encased in a polar lipid monolayer (**Figure 2-2**). Chylomicrons are considered to be triglyceride-rich particles because they are approximately 88% TG (Olson, 1998). In addition, they carry between 2-5% of the plasma cholesterol pool (Mamo & Proctor, 2002). The outer layer, a phospholipid bilayer, is embedded with unesterified cholesterol and apolipoproteins characteristic of the chylomicron. Apo-B48 is a structural protein associated uniquely with the chylomicron and it is essential for chylomicron assembly. Apo-B48 is synthesized from the same gene as the hepatically derived apolipoprotein-B100 (apo-B100), however a stop codon approximately halfway (48%) past the amino terminus produces a ~241 kilodalton protein. In humans and higher order mammals, the stop codon is inserted into intestinal mRNA which accounts for its exclusive secretion from the intestine.

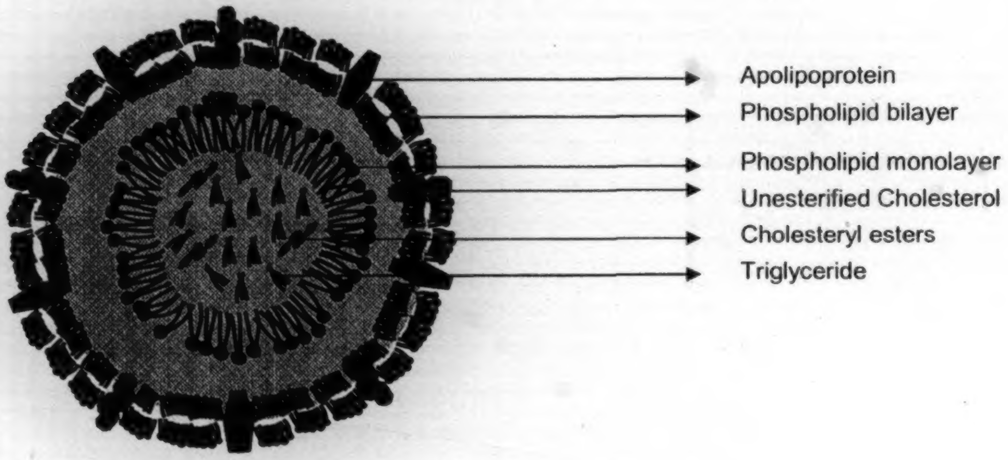
Apolipoprotein
Phospholipid bilayer
Phospholipid monolayer
Unesterified Cholesterol
Cholesteryl esters
Triglyceride

Figure 2-2 Diagram of a native chylomicron particle

2.3.3 Chylomicrons and cholesterol metabolism

2.3.3.1 The intestine: Inherently, the concentration of chylomicron particles in plasma, particle size and number depends upon the prandial state of the individual. Fasting chylomicron concentration is the homeostatic result of both intestinal production and hepatic clearance. However, even in the absence of dietary fat, native chylomicrons are constitutively produced and secreted from the intestine. Increased secretion of chylomicrons becomes stimulated upon ingestion of a fat-containing meal (Chan *et al.,* 2002a). Further, both the production and secretion of chylomicron particles may be up-regulated in an insulin resistant state suggesting a potential role for chylomicrons in chronic diseases (Haidari *et al.,* 2002, Lewis *et al.,* 2005, Lewis *et al.,* 2004).

2.3.3.2 In circulation: From the intestine, chylomicrons travel directly to the lymphatic collecting duct and pass into circulation. Under normal conditions, native chylomicrons are rapidly hydrolysed within the circulation by lipoprotein lipase, an enzyme located on the surface of endothelial cells in adipose tissue and muscle (Redgrave, 2004). With its cofactor apolipoprotein C-II (apo C-II), lipoprotein lipase hydrolyzes the chylomicron-releasing TG, free fatty acids and free cholesterol to become a smaller, dense chylomicron-remnant (CR). Investigators have hypothesized that the actions of apo C-II may ensure that chylomicrons have sufficient time to be hydrolysed in plasma (Shelburne *et al.,* 1980; Windler *et al.,* 1980).

Chylomicron remnants are considerably smaller than the native chylomicron and vary in size from 45 and 60 nm (Redgrave, 2004). In addition, their composition is slightly different (**Table 2-3**). Compared to the native particle, CRs are TG-depleted, have less phospholipid, and a greater proportion of cholesterol ester (Cooper, 1997). Due to their size, CRs are potentially atherogenic and plasma concentrations are clinically important. Chylomicron remnant concentrations are elevated in insulin resistant, viscerally obese men (Chan *et al.,* 2002a; Chan *et* *al.*, 2002b; Couillard *et al.*, 2002). Efficient hepatic clearance of chylomicron remnants is pivotal to minimizing their atherosclerotic potential.

Table 2-3 Comparison and contrast of native and remnant chylomicron particles

Characteristic	Native Chylomicron	Remnant chylomicron
Diameter (nm)	200-1000	45-60
Apolipoproteins	B48, CI, CII, CIII,E	B48, E
Composition		
Triglyceride (%)	88	70
Cholesterol Ester (%)	2-5	13
Phospholipid (%)	3-8	11
Protein (%)	2	6

2.3.3.3 Clearance: Following hydrolysis chylomicrons are cleared by several receptor-mediated processes, including the LDL receptor, LDL-receptor related protein and a phospholipolysis-dependent pathway (Yu *et al.*, 2000). Chylomicron clearance is influenced by a number of factors including the apolipoprotein E (apo E) to apo C ratio, particle size (it must be able to fit through the fenestrae of the endothelium), abundance of the LDL-receptor, and the LDL-related receptor protein (Cooper, 1997). Small chylomicron and CR particles gain entry into the hepatocyte in the space of Disse, a space between the endothelial cells lining the hepatic sinusoids and the hepatocytes themselves. CR-associated apo E is the primary protein responsible for CR clearance (Cooper *et al.*, 1982; Sherrill *et al.*, 1980). Apo E interacts with the apo-B100/apoE receptor on the surface of the hepatocyte. It has been shown that apo E has a higher affinity for the apo-B100/apoE receptor compared to apo-B100 (Innerarity & Mahley, 1978; Mahley & Innerarity, 1983) and the higher affinity is thought to be one of the factors that accounts for why chylomicrons are more rapidly cleared than LDL particles (Proctor *et al.*, 2004). The rate of chylomicron clearance is important since delayed clearance is linked to IR, obesity, and accelerated atherogenesis (Mamo & Proctor, 2002). For example, remnant removal has been shown to be delayed in people prone to premature atherosclerosis including, individuals on dialysis (Hirany *et al.*, 2000; Karpe *et al.*, 1994) or with T2DM (Chen *et al.*, 1993) despite normal levels of LDL-C and other traditional CVD risk factors. Substantial clinical evidence supports the hypothesis that delayed CR clearance itself may accelerate atherogenesis.

2.3.4 Chylomicrons in atherosclerosis: The role of chylomicrons in the initiation and progression of atherosclerosis is best explained by the "response to retention" hypothesis. Since the 1970s, it has been proposed that the initial step of atherosclerosis is the entrapment and retention of lipoproteins in the subendothelial space (Camejo *et al.*, 1975; Iverius, 1972; Schwenke & Carew, 1989a; Schwenke & Carew, 1989b; Vijayagopal *et al.*, 1981). More specifically, it is thought that atherogenic lipoproteins gain entry through the vessel wall via transcytosis (vesicular transport across the cell from the plasma to the subendothelial space) (**Figure 2-3**) (Simionescu & Simionescu, 1991a; Simionescu & Simionescu, 1993; Simionescu & Simionescu, 1991b). Lipid particle transport via transcytosis is limited by particle size (maximum: 70 nm) which excludes nascent chylomicrons, but allows both CRs (40-60nm) and LDL (27 nm) particles to pass through (Proctor & Mamo, 1998). Upon entry into the subendothelial space, positively charged amino acid residues on the apolipoproteins can interact with the negatively charged amino acids on a component of the extracellular matrix which slows down the rate of permeability. Triglyceride-rich lipoproteins (TRL) can bind and become retained in the intima (Hurt-Camejo *et al.,* 1997) by way of their associated proteoglycans (Flood *et al.*, 2002). Proteoglycans, a component of the extracellular matrix, are a macromolecule with a core protein and polysaccharide side chains called glycosaminoglycans. Once entrapped by the proteoglycans, the cholesterol contained in the CRs can be also oxidized (Yu *et al.*, 2000). Importantly, since CRs contain 42 times the amount of cholesterol compared to LDL-C, CRs may deliver more cholesterol to arterial tissue (Proctor *et al.,* 2002).

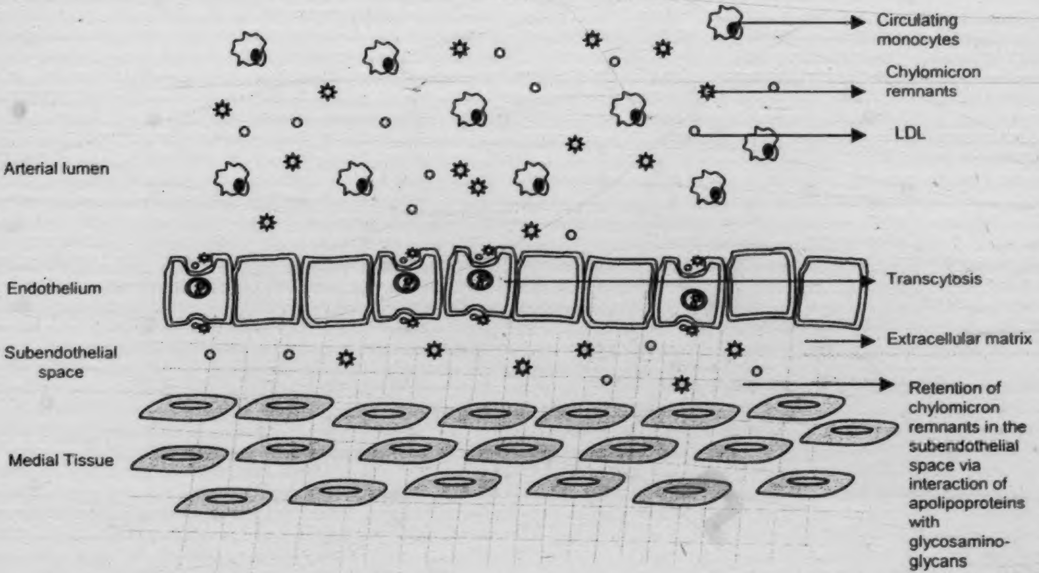
Circulating monocytes
Chylomicron remnants
LDL
Arterial lumen
Endothelium
Transcytosis
Subendothelial space
Extracellular matrix
Retention of chylomicron remnants in the subendothelial space via interaction of apolipoproteins with glycosamino-glycans
Medial Tissue

Figure 2-3 Diagram of the initiation of atherosclerosis, lipid deposition, and entrapment in the vessel wall

The endothelium begins to express surface adhesion molecules, such as endothelial cell adhesion molecule-1 (ECAM-1) and vascular adhesion molecule-1 (VCAM-1), during the initial stages of the atheroma or lipid deposition to allow macrophages to extravagate (Cybulsky *et al.,* 2001; Li *et al.,* 1993a; Li *et al.,* 1993b) (**Figure 2-4**). Interestingly, chylomicrons have also been shown to have a chemotatic effect on human-derived monocytes *in vivo* (Proctor, 2000) and to induce expression of ECAM-1 and VCAM-1 on human endothelial cells *in vitro* (Moers *et al.,* 1997). Once bound to surface adhesion molecules, monocytes extravagate between endothelial cells into the subendothelial space and migrate towards the lesion in response to chemoattractant molecules released from the site of the lesion. At the site of the lesion, monocytes differentiate to form macrophages. Activated macrophages endocytose oxidized lipid and once saturated, differentiate into foam cells and form the fatty streak (**Figure 2-5**) (Libby *et al.,* 2002). Chylomicrons contain lower levels of vitamin E and platelet-activating factor acetylhydrolase, compared to other lipid subfractions which may increase their propensity for oxidation (Lee *et al.,* 1999).

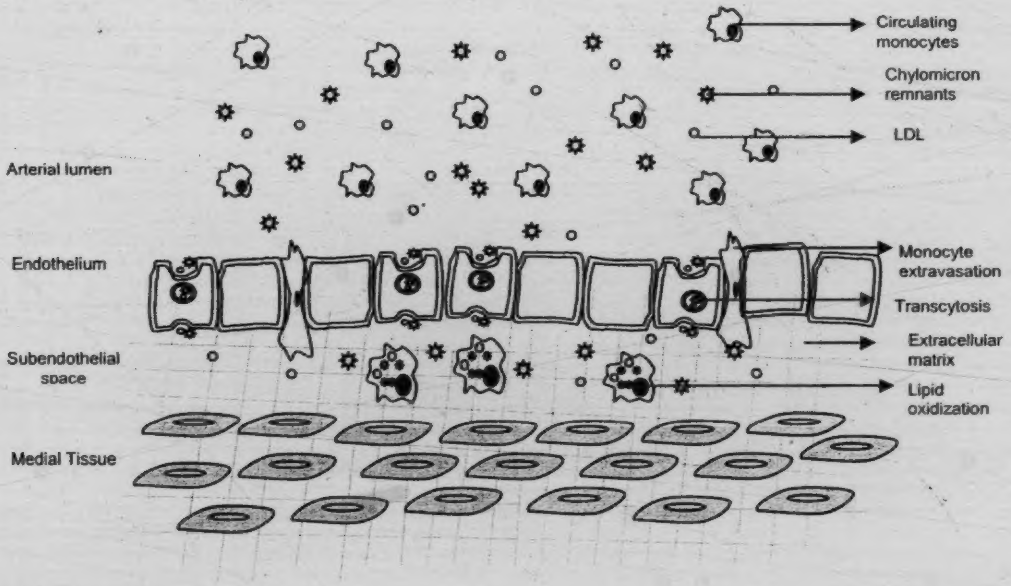
Circulating monocytes
Chylomicron remnants
LDL
Arterial lumen
Endothelium
Monocyte extravasation
Transcytosis
Subendothelial space
Extracellular matrix
Lipid oxidization
Medial Tissue

Figure 2-4 Diagram of leukocyte recruitment and extravasation through the endothelial wall

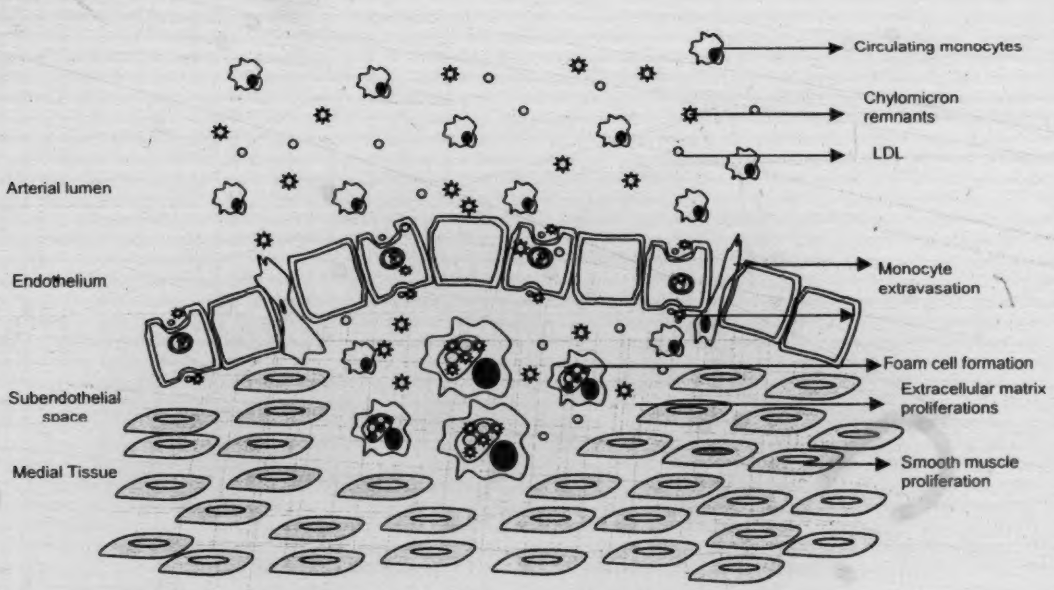
Circulating monocytes
Chylomicron remnants
LDL
Arterial lumen
Endothelium
Monocyte extravasation
Foam cell formation
Extracellular matrix proliferations
Subendothelial space
Smooth muscle proliferation
Medial Tissue

Figure 2-5 Diagram of the advanced fatty streak.

As the atheroma progresses, T-cells release hydrolytic enzymes like metalloproteases which lead to local necrosis or cell death (Hansson *et al.*, 1996; Galis *et al.*, 1994). Accumulated necrotic inflammatory cells, smooth muscle cell proliferation and fibrous tissue formation all restructure the lesion and form a fibrous cap (**Figure 2-6**) (McGill *et al.,* 1963). It has been shown that TRLs, which included CRs, may increase thrombosis, (dislodgement of an aggregation of platelets and fibrin) via stimulation of endothelial cells (Eriksson *et al.,* 1998). Eventually the plaque will become so structurally compromised that either a physical disruption to the plaque, hemorrhage into the plaque or luminal thrombosis occluding the vessel will occur resulting in ischemia, heart attack or stroke (Schroeder & Falk, 1996).

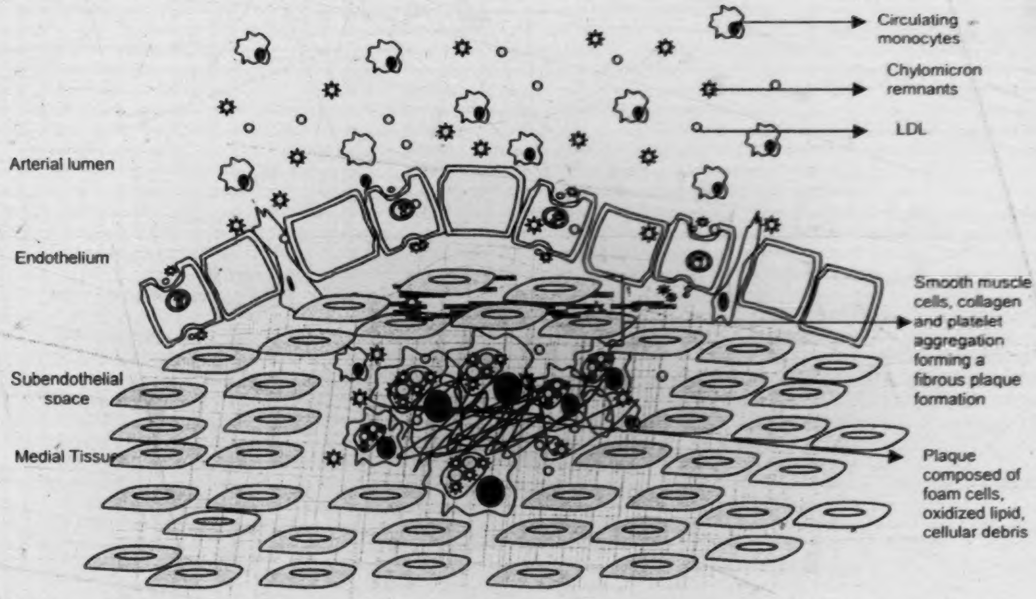
Circulating monocytes
Chylomicron remnants
LDL
Arterial lumen
Endothelium
Smooth muscle cells, collagen and platelet aggregation forming a fibrous plaque formation
Subendothelial space
Medial Tissue
Plaque composed of foam cells, oxidized lipid, cellular debris

Figure 2-6 Diagram of the fibrous plaque.

In summary, significant advancements in chylomicron research have altered the long held belief that the lipoprotein has no role in atherosclerosis. It is now known that chylomicrons, particularly in their remnant form, have a direct role in atherosclerosis. Delayed chylomicron remnant clearance increases delivery of TG and cholesteryl esters to the vessel wall, accelerates the progression of CVD (particularly in insulin resistant, obese individuals (Chen *et al.,* 1993)), and suggests a role for delayed clearance in chronic disease states.

2.3.5 Chylomicrons and clinical studies: Although findings are equivocal (Sharrett *et al.,* 1995; Valero *et al.,* 2005), most studies report that men and women with CAD exhibit greater fasting and postprandial concentrations of chylomicrons compared to healthy subjects, even when the patients are otherwise normolipidemic (Cohn *et al.,* 1999). For instance, Mero *et al.* (2000) conducted a case-control study with 43 subjects with severe (n = 27) and mild (n = 16) CAD to examine differences in the post-prandial chylomicron response. Coronary artery disease severity was determined by a coronary angiogram and apo-B48 was measured in every ultracentrifugation subfraction containing chylomicrons (Svendberg flotation rate (Sf) > 400) to intermediate (IDL) (Sf 12-20). Although there were no differences between groups, post-prandial apo-B48 was persistently elevated in the severe CAD group, compared to a normal healthy control reference group (n = 10) (3.5 mg/L *versus* 1.0 mg/L); especially in the Sf 40-600 fraction. Moreover, Rajanatman *et al.*, (1999) demonstrated higher fasting apo-B48 (measured from the TRL fraction) in post-menopausal women with CAD (n = 24) compared to women without CAD (n = 30) (3.10 ± 0.39 mg/L *versus* 1.67 ± 0.22 mg/L; p < 0.001, respectively) despite similar serum TG, TC and apo B concentrations. In addition to elevated fasting apo-B48, subjects with CAD demonstrated significantly higher post-prandial lipemia compared to controls, indicated by the higher incremental area under the apo-B48 concentration curve (20.6 ± 2.8 *versus* 11.0 ± 1.2; p < 0.01).

A case control study of normolipidemic adults with (n = 85) and without (n = 85) CAD (Weintraub *et al.,* 1996) examined differences in chylomicron concentration. Using the retinyl palmitate enriched-fat meal, Weintraub *et al.* demonstrated that with normal LDL-C, HDL-C and TG levels, subjects with CAD displayed elevated fasting and postprandial retinyl esters (RE) in the CR fraction compared to controls. The CR fraction area-under-the-curve was greater in CAD, even after adjustment for TG and HDL-C (23.4 ± 15.0 *versus* 15.3 ± 8.9; $p < 0.001$). McNamara *et al.* (2001) reported similar results in their cross-sectional study of 1184 women in the Framingham Heart Study (cycle 4). Remnant-like lipoprotein cholesterol ((RLP-C: containing both intestinally and hepatically derived remnant particles); 0.22 ± 0.10 *versus* 0.19 ±0.16 mmol/l; $p < 0.0001$) and RLP-TG (0.32 ± 0.35 *versus* 0.25 ± 0. 72 mmol/l; $p < 0.0002$)) were significantly higher in the women with CHD (n = 84) compared to those without (n =1100), respectively. Furthermore, RLP-C ≥75th percentile predicted the presence of CAD (OR: 2.27; CI: 1.37-3.77).

There is accumulating evidence that elevated chylomicron concentrations can predict a CVD event. A longitudinal study (~ 30 years) (Imke *et al.*, 2005) of Hawaiian men divided participants into two groups: those with (n = 164) CAD and those without (n = 992). Imke *et al.* found that RLP-C and RLP-TG predicted CHD occurrence independent of HDL-C, LDL-C and non-lipid CVD risk factors (including age, BMI, smoking, alcohol intake, physical activity, systolic blood pressure, fasting glucose, history of diabetes and antihypertensive medication) .

Collectively, there is clinical evidence that individuals with CAD may have elevated fasting apo-B48 and post-prandial lipemia, despite otherwise normal LDL-C (Cohn *et al.*, 1999; Mero *et al.*, 2000, Weintraub *et al.*, 2006). This is of particular concern since LDL-C is the lipid measure used most often in adults to assess CVD risk and the pediatric CVD risk profile is largely based on adult data. Given that chylomicrons are directly involved in the initiation of atherosclerosis and since overweight children often have normal LDL-C, a greater understanding of chylomicron metabolism in pediatrics is critical. Due to the absence of data on chylomicrons in children, this type of research is ground-breaking; however, the progression of this field has been limited due to the technical challenge of accurately quantifying chylomicron (via apo-B48) concentrations.

2.3.6 Chylomicrons and methodology: Accurately measuring apo-B48 in plasma relies upon the use of a method that is able to separate the apo-B48 sufficiently from its major contaminant apo-B100 containing particles. The method must also capture both large nascent chylomicrons and small CRs (Jackson & Williams, 2004) (**Table 2-4**). Classically, samples have been derived through density gradation and sequential ultracentrifugation. Chylomicrons and very low density lipoproteins (VLDL) are isolated in the total triglyceride-rich fraction (density (d) = 1.006 g/ml). LDL and some CRs are found in the $d < 1.063$ g/mL fraction. Increasing the speed and time of ultracentrifugation produces TRL-rich subfractions which include $S_f < 400$ (nascent chylomicrons), S_f 20-400 (heavy VLDL, IDL), S_f 12-20 (IDL) and S_f 0-12 (LDL and CRs) (Campos *et al.*, 2005; Couillard *et al.*, 2002; Jackson & Williams, 2004; Karpe *et al.*, 1994; Tomkin & Owens, 2001)[5]. The main limitations of using total fractions isolated from density gradation and ultracentrifugation are that the density fractions are contaminated with apo-B100-containing particles (VLDL, IDL, and LDL) and not all apo-B48 containing particles are found in one subfraction. As well, chylomicrons and CRs are found in two distinct subfractions. (Hussain *et al.,* 2005). To minimize loss of chylomicrons, often multiple subfractions are included in measurement; however apo-B100 contamination can still be problematic.

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[5] Although the techniques are positively and significantly correlated, the measurement of lipoproteins using density grading, ultracentrifugation, SDS-PAGE and ELISAs differs from lipoprotein cholesterol measurements performed in the clinical setting.

To isolate chylomicrons and CRs from these fractions after ultracentrifugation, apo-B48 is isolated and measured based on molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or gel filtration (Bjorkegren *et al.,* 2002) (**Table 2-4**). Following gel filtration, protein can be visualized directly on the gel using dye or staining or via immunoblotting. Immunoblotting is a more precise method because it uses specific antibodies to apo-B48 (Jackson & Williams, 2004). Due to the additional steps involved in the immunoblotting process, it is a distinct method from dye / staining.

Other proxy methods for measuring chylomicrons in circulation include measuring remnant lipoprotein cholesterol (RLP-C), a cholesterol subfraction composed of remnant particles. This subfraction contains apo E, is TG-depleted and is relatively cholesterol dense. RLP-C can be separated by ultracentrifugation (found in the 1.006 g/ml < *d* < 1.019g/ml or S_f 40-60 fraction), SDS-PAGE, or by immunoaffinity chromatography. However, RLP-C has been reported to contain both hepatically-derived apo-B100 and intestinally-derived apo-B48-containing lipoproteins; studies are not able to separate the actions of each (Lemieux *et al.*, 1998, Cohn, 2006).

Table 2-4 Summary of description and limitations of methods used to measure chylomicrons in human studies

Method	**Specifics**	**Limitations**
Density grading	*d* < 1.063 g/mL and 1.006 g/mL	Contamination by VLDL and LDL particles.
Ultracentrifugation	Sf < 400, 0 < Sf < 12	Contamination by VLDL and LDL particles.
SDS-PAGE	Molecular weight (241 KDa)	Multiple-step protocol and less sensitive.
Gel stain or dye	Coumassie blue	Non-specific and less sensitive.
Immunoblotting	Polyclonal or monoclonal antibody	Time-consuming, but more sensitive.
RLP-C	Immunoaffinity chromatography with monoclonal antibodies	Contamination by hepatically-derived remnant lipoproteins.
Retinyl palmitate	Measures incorporation of lipid in chylomicrons	Retinyl esters may transfer to other lipid subfractions.
ELISA	Competitive binding with apo-B48 antiserum	Not commercially available.
Breath Test	C^{13} labelled-emulsion injection	Not commercially available.

Abbreviations: d (density), VLDL (very low density lipoprotein), LDL (low density lipoprotein), Sf (Svedberg flotation rate), SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), KDa (KiloDalton), ELISA (enzyme linked immunoasbsorbent assay), C (carbon)

Measurement of REs after ingestion of a retinyl palmitate (vitamin A)-enriched meal is another surrogate marker for chylomicrons and their remnants. Since ingested lipophylic vitamin A molecules will incorporate into chylomicron particles, measuring RE in plasma can be a surrogate measure of chylomicron concentration. Some investigators have reported transfer of REs (via vitamin A) from chylomicron particles to other lipoproteins; consequently REs may be a non-specific marker of chylomicrons (Cohn, 2006). More recent methods used to measure chylomicrons include (1) enzyme linked immunoabsorbent assays

(ELISA), where competitive binding with an apo-B48 antiserum can be used to measure apo-B48 in the sample (Kinoshita *et al.,* 2005; Lorec *et al.,* 2000; Lovegrove *et al.,* 1996; Sakai *et al.,* 2003; Valero *et al.,* 2005) and (2) a breath test (Chan *et al.,* 2002b; Dane-Stewart *et al.,* 2003; Martins & Redgrave, 1998; Redgrave *et al.,* 2001; Watts *et al.,* 2001). The breath test measures the kinetics of an emulsion injection that mimics chylomicron concentration to measure chylomicron clearance and metabolism.

Although RLP-C immunoaffinity assays, ELISAs, and breath tests are gaining in popularity due to the simplicity and convenience of the techniques, commercial kits are not yet available. In addition breath tests and RLP-C assays do not measure apo-B48 with sufficient specificity to isolate the effects of chylomicrons (Cohn, 2006). Variations in protocol and antibodies also result in large variations in reported apo-B48 concentrations. SDS-PAGE immunoblotting remains the research gold-standard, upon which new methods are tested and validated. In order to circumvent some of the disadvantages of ultracentrifugation, and capitalize on the advantages of the SDS-PAGE immunoblotting method, our laboratory has developed a unique SDS-PAGE-immunoblotting technique. The method, first used by Smith *et al.* (1997), measures apo-B48 directly from whole plasma, and eliminates the labour-intensive ultracentrifugation process. Protein bands are visualized on a membrane using enhanced chemiluminescence (ECL) which allows for more accurate measurement of apo-B48 concentrations (Vine *et al.*, 2007). Despite the precision of the SDS-PAGE immunoblotting method, there remains substantial variability in the concentrations of apo-B48 reported by laboratories using this modified technique (**Table 2-5**). It is possible that other factors such as the use of monoclonal *versus* polyclonal antibodies, small sample size and gender variations have contributed to measurement variability. In order to minimize variability in our study, plasma samples were run in triplicate and measured concentrations had to be within 10% of each other. Despite the variability, apo-B48 is consistently shown to be elevated in obese and / or insulin resistant men and women compared to lean and / or normolipidemic controls using this technique suggesting that there is a robust link between excess weight, IR, and elevated apo-B48.

Table 2-5 Summary table of studies reporting apo-B48 concentrations using the SDS-PAGE immunoblotting technique.

Reference	Subjects	n (M/W)	Age (years)	BMI (kg/m^2)	Apo-B48 Concentration (mg/L)
Dane-Stewart *et al.*, 2003	Non-diabetic	0/24	60 (2)	25.9 (0.9)	13.0 (0.92)
	Diabetic	0/14	60 (1)	25.9 (0.7)	16.4 (1.2)
Chan *et al.*, 2002	Non-obese	10/0	53.1 ± 9.0	24.8 ± 2.9	12.3 ± 2.8
	Obese	48/0	53.5 ± 9.0	33.6 ± 4.1	24.3 ± 8.8
Dane-Stewart *et al.*, 2001	FH controls	8/7	51 (2)	27.4 (1.0)	12.8 [7.3 – 28.6]
Mamo *et al.*, 2001	Non-obese	14 (?/?)	46.3 (2.6)	24.7 (0.5)	12.7 (1.7)
	Obese	21 (?/?)	48.8 (1.9)	35.8 (0.9)	31.5 (7.5)
Redgrave *et al.*, 2001	Non-obese	16 (?/?)	44	Not reported	6.9 (1.1)
Smith *et al.*, 1999	Non-obese	?/0	18-65	Not reported	6.9 (1.1)
Mamo *et al.*, 1998	Non-obese	5 (?/?)	35 (7)	Not reported	2.0 (0.5)

Abbreviations: M (men), W (women), FH (familial hypercholesterolemia)
Data expressed as mean (standard error of the mean), mean ± standard deviation or mean [range].
All the studies cited in this table, with the exception of Redgrave 2001 and Mamo 1998 (not specified) used the same polyclonal antibody from DakoCytomation, Germany (catalogue number Q0497).

2.3.7 Conclusion: Lack of standardization in the methods used to measure chylomicron concentrations is one of the major limitations of its study in humans.

However, accurate and comprehensive measures of chylomicrons, such as our modified SDS-PAGE immunoblotting technique, will help to improve the reliability of chylomicron measurement. Also, despite variations in methodology there is consistent reporting of elevated chylomicrons / apo-B48 in subjects with CVD who are otherwise normolipidemic. This suggests that additional study of chylomicrons as a measure of CVD risk is needed, particularly in the pediatric population.

2.4 General Conclusion:

If unabated, the growing prevalence of overweight, IR and dyslipidemia in Canada will result in an exponential growth in CVD-related morbidity and mortality. Due to the chronic and progressive nature of atherosclerosis, early (pediatric) and accurate detection is essential for prevention and / or treatment. Further, atherosclerosis can progress for decades asymptomatically, so it is important that the best indices of the presence and progression of atherosclerosis are identified and applied. Currently, the lipid profile used to assess CVD risk does not include a measure of chylomicron concentration (in adults or children); however, chylomicrons are preferentially retained in the subendothelial space and a substantial contributor of intimal cholesterol deposition. Clinically, this becomes pertinent for individuals who have other lipid concentrations within the normal range, especially LDL-C, and yet have persistent and substantial impairment in the metabolism and clearance of chylomicrons and their remnants. Atherosclerosis is a disease that begins in childhood and measurement of chylomicrons at this time in life may be highly relevant in the context of CVD risk. Consequently, chylomicrons (via apo-B48 detection) offer a unique opportunity to better understand and identify the potential risk of CVD in overweight children. With these issues in mind this thesis was the first to examine the chylomicron concentration in overweight, pre-pubertal children. In doing so, fasting chylomicron levels were characterized and their relationship to classic CVD risk factors in the pediatric population was explored.

Chapter 3 Methods

3.1 Participants

3.1.1 Recruitment: Children were recruited for this cross-sectional study primarily by physician referral from the Capital Health Region in Edmonton, Alberta. A number of alternative methods including posters, newspaper and newsletter advertisements, local newspaper articles, radio and television interviews, recruitment through the Stollery Children's Hospital (SCH) Pediatric Ambulatory Clinic, and word-of-mouth were also used to recruit children. Each child underwent pre-screening (by phone or in person) to establish eligibility for the study (**Appendix A**[6]).

Sixty-one children were referred by a physician to the study. Of these, 8 could not be contacted, 12 were screened by phone and 41 were screened in person. No children were enrolled from phone interviews (n = 12; all declined for a variety of reasons, such as too much time commitment, holidays, spouse refused to consent and did not satisfy inclusion criteria). All 40 participants were enrolled in the study based on in-person interviews. One child from in-person interviews did not meet inclusion criteria.

3.1.2 Inclusion and exclusion criteria: The inclusion criteria included: 6-11 years of age, pre-pubertal (Tanner stage 1), and overweight (as defined by a BMI ≥95th percentile for the child's age and height; Center of Disease Control and

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[6] Forms were authored by Dr. Mary Jetha.

Prevention (CDC) 2000 growth charts (Kuczmarski *et al.*, 2000)). Children were also screened for the following exclusion criteria: any underlying confounding medical conditions (such as chronic lung disease, congenital heart disease, hepatic renal impairment or neurological disorder), any known syndrome associated with weight or altered glucose / lipid metabolism (such as Down or Prader-Willi syndrome), a history of brain tumour, injury, irradiation or surgery that could influence weight or glucose / lipid metabolism, history of systemic steroids, contraindications to MRI and current medication use that could influence weight or glucose / lipid metabolism.

3.1.3 Visits: Visit one was conducted at the SCH Pediatric Ambulatory Clinic. The study was explained in detail to both parents and children and consent and assent forms, respectively, were completed (**Appendix B and C**[7]). At this time, the physician verified both the inclusion and exclusion criteria, conducted anthropometric measurements and completed the physical exam. Visits two and three (oral glucose tolerance test (OGTT) / magnetic resonance imaging (MRI) / dual x-ray energy absorptiometry (DXA)) were scheduled at the conclusion of the first visit and separated by a minimum of 7 days and a maximum of 60 days. At visit three, children received four passes for Edmonton recreation centres as tokens of appreciation. **Figure 3-1** shows the sequence of visits / events for participants from recruitment, enrolment and study completion.

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[7] Forms were authored by Dr. Mary Jetha.

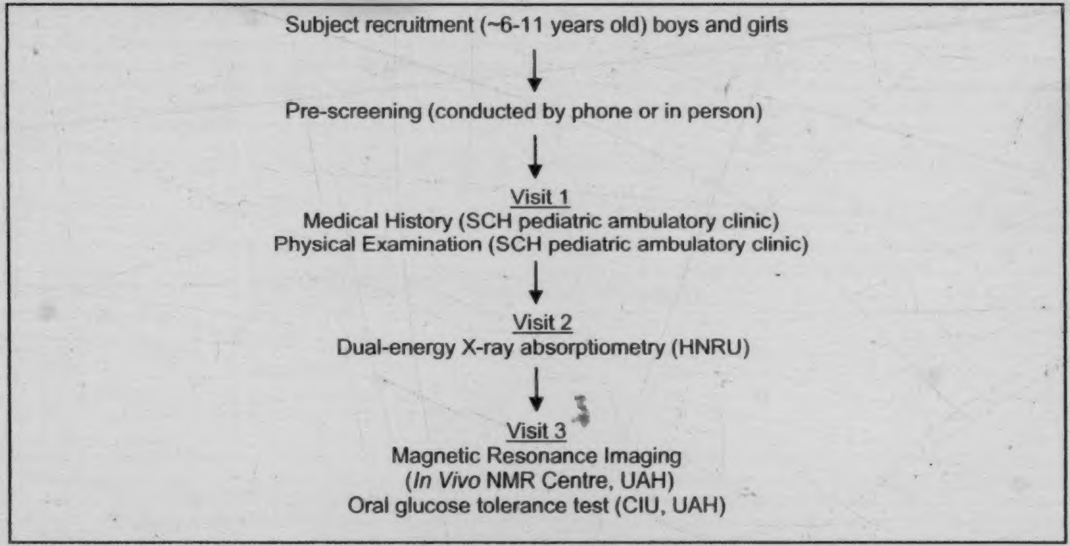
Subject recruitment (~6-11 years old) boys and girls
Pre-screening (conducted by phone or in person)
Visit 1
Medical History (SCH pediatric ambulatory clinic)
Physical Examination (SCH pediatric ambulatory clinic)
Visit 2
Dual-energy X-ray absorptiometry (HNRU)
Visit 3
Magnetic Resonance Imaging
(In Vivo NMR Centre, UAH)
Oral glucose tolerance test (CIU, UAH)

Figure 3-1: Summary of subject visits

Abbreviations: SCH (Stollery Children's Hospital), HNRU (Human Nutrition Research Unit), NMR (nuclear magnetic resonance), CIU (Clinical Investigations Unit), UAH (University of Alberta Hospital)

3.2 Procedures

3.2.1 Anthropometry: (Visit 1) Height (to the nearest 0.1 cm) was measured in triplicate with a wall mounted stadiometer (Holtain Limited, United Kingdom). Children were asked to remove their shoes, place their heels together against the wall and stand tall. Each of the three measures was recorded and the average calculated. Weight was measured in triplicate (to the nearest 0.1 kg) using an electric medical scale (Healthometer, Bridgeview, IL). Each of the three weight measures was recorded and the average calculated. BMI was subsequently calculated using the equation: BMI = weight in kilograms / (height in metres)^2. Waist circumference (to the nearest 0.1 cm) was measured in the standing position and at the level of the umbilicus after exhalation. Three measurements were taken with a standard non-stretch tape measure and the average was calculated. All anthropometric measurements were performed by one physician trained in performing anthropometric measurement.

3.2.2 Physical exam: (Visit 1) Resting SBP and DBP were taken from the right arm with a standard mercury sphygmomanometer and a stethoscope after 2 minutes of quiet sitting. Cuff size was matched to the upper right arm circumference. Three BP measurements were taken one minute apart and the measures were recorded and averaged. SBP and DBP percentiles were determined using the NHLBI 2004 blood pressure charts for children and adolescents (NHLBI, 2004). Pre-pubertal status (Tanner stage) was verified by physical exam according to the criteria defined by Marshall and Tanner (Marshall & Tanner, 1969; Marshall & Tanner, 1970).

3.2.3 DXA: (Visit 2) Body composition was measured using the General Electric LUNAR Prodigy High Speed Digital Fan Beam X-Ray-Based Bone Densitometer in the Human Nutrition Research Unit (HNRU) in the Department of Agricultural, Food and Nutritional Science, University of Alberta. All measurements were performed using a standardised procedure by a trained medical X-ray technologist. Each participant's clothing was inspected and approved by the technologist. A whole body scan was performed (total scan time: 5 minutes). Whole body and regional (i.e., trunk) analysis provided absolute values for fat mass (FM) (kilograms), lean tissue mass (LTM) (kilograms) and percent lean tissue (%BL). Subsequently, percent body fat (%BF) was calculated from the equation: 100% - %BL.

3.2.4 *OGTT*: (Visit 3) Participants completed an overnight fast (aside from water, no food or drink was consumed, after 8:00pm the previous evening) and refrained from vigorous physical activity the day prior to the OGTT. Fasting from food / drink and physical activity was verified by a questionnaire delivered by the research nurse the morning of the OGTT. Children were admitted as outpatients to the Clinical Investigations Unit (CIU) at the University of Alberta Hospital (UAH). An intravenous catheter was inserted into the intracubetal vein of the right arm and a 12 mL baseline (time: 0 minutes) blood sample was drawn. Following baseline sampling, participants consumed a 296 mL (75 g glucose), carbonated orange-flavoured sugar drink (Trutol 75, Nerl Diagnostics, East Providence, RI) within 5 minutes. Blood samples (4 ml) were collected from the intravenous catheter at 15, 30, 45, 60 90 and 120 minutes for biochemical analysis. All blood samples (except one ethylenediamine tetraacetic acid (EDTA)-containing vacutainer and one serum separating-vacutainer per time point) were sent to the UAH Laboratory (Capital Health, Edmonton, AB) for analysis. An additional fasting blood sample for apo-B48 analysis as well as the EDTA-containing and serum separating vacutainers, not intended for the UAH laboratory, were collected and transported to the HNRU according to standard biosafety procedures at the HNRU. Serum and plasma samples were separated by centrifugation in a Jouan CR4.22 Refrigerated Centrifuge (Canberra Packard

Canada Ltd.) at 3000 rpms (Radius: 172 mm; Time/Interval (1/0):1; Time: 10 minutes; Temperature: 4°C; Temperature Compensation: 4°C; Acceleration Rate: 9; Brake: 9; RP/RCL(1/0):1). Plasma and serum were collected, aliquoted into 1.5 mL eppendorf tubes and stored at -80 °C for future analysis.

3.2.5 MRI

3.2.5.1 Image acquisition: (Visit 3) MR image acquisition took place at the UAH *In Vivo* Nuclear Magnetic Resonance (NMR) Centre. Children wore clothes without metal (zippers, clasps) and removed all metal jewellery. If clothes were considered unacceptable by the MRI technologist, the participants changed into a hospital gown. Children were positioned on the table of the 1.5T Siemens Sonata MRI machine (Erlangen, Germany). The radiofrequency coils (CP Body Array Extender and CP Body Array Flex, Siemens, Erlangen, Germany) were placed on the participants (between the chest and groin) and secured with straps. Children were handed an emergency call button which could be used at any time to alert the technologist to stop the procedure. The MRI acquisition protocol was designed exclusively for our study by Drs. Christian Beaulieu and Richard Thompson (Department of Biomedical Engineering, UAH). Briefly, image acquisition began with three localizer scans (coronal, sagittal and frontal) used to center the images on the participant's body at the fourth lumbar vertebra. Subsequently, three sequences (HASTE [Half-fourier Acquisition Single shot Turbo Spin Echo], HASTE Water SAT [HASTE Water Suppression] and FISP [fast imaging with steady precession]) were run. Twenty 0.6cm-thick transaxial images were acquired for each of these sequences. Next, the table was moved inward 120mm (thickness of 20 slices) and the pelvic region was scanned using the three sequences. Finally, the table was moved outward 240 mm (thickness of 40 slices) and the chest region was scanned. In total, 60 contiguous slices (no gap) for each sequence were acquired, encompassing the chest, abdominal and pelvic regions. Each set of localizer scans required breath holding for 13-14 seconds and each sequence of transaxial scans required breath holding for 15 seconds. Total scan time was approximately 30 minutes. All images were acquired with a rectangular field of view (FoV) (FoV phase = 75%). Only HASTE Water SAT images were used to calculate tissue volumes because the water suppression in the images helped to better differentiate between lean tissue and fat tissue. Other images collected were used to clarify ambiguities when identifying organs and other anatomical structures. See **Table 3-1** for details of the parameters for each sequence.

3.2.5.2 Image analysis: Image analysis was performed using the MRI image analysis program *Slice-O-Matic* (Version 4.3, Tomovision, Montreal, QC) to quantify total visceral adipose tissue (VAT) and subcutaneous abdominal adipose tissue (SAAT) area. Only images containing abdominal VAT (approximately from the eleventh thoracic vertebrae to the top of the femoral heads) were included in image analysis. Using *Slice-O-Matic*, VAT was assigned a red tag color and SAAT was assigned a blue tag color. Preliminary image analysis was performed in the Mathematical Morphology mode to segment the image according to tissue by computing the watershed of the gradient of the image (see yellow lines in **Figure 3-2**).

Table 3-1 Details of parameters for each sequence (HASTE, HASTE Water SAT and FISP) used in MRI acquisition

Sequence	**HASTE (localizers: frontal, coronal, sagittal)**	**HASTE (transaxial)**	**HASTE Water SAT (transaxial)**	**FISP (transaxial)**
Number of slices	7	20	20	20
Slice thickness (mm)	6	6	6	6
Distance factor (mm)	12	0	0	0
FoV read (mm)	384	400	400	400
FoV phase (%)	75	75	75	75
Image matrix (mm)	144 X 192	144 x 192	144 x 192	144 x 192
TR (ms)	600	700	700	600
TE (ms)	81	19	79	1.48
Flip angle (degrees)	130	130	130	65
Fat suppression	None	None	None	None
Water suppression	None	None	Yes	None
TA (s)	13	15	15	15

Abbreviations and: FoV (Field of view),), TE (Echo time), FISP (fast imaging with steady precession), HASTE (half-Fourier acquisition single shot TurboSE), TI (inversion time), TR (repetition time), TA (acquisition time)

Definitions: Image matrix: measure of pixilation, TE: time between the excitation pulse of a sequence ant the resulting echo used as the MR signal, determines image contrast, TI: interval between 180° pulse and 90° excitation pulse in an inversion recovery sequence, TR: time between two excitation pulses, helps to determine contrast, TA: measurement time for a complete data set, distance factor: gap between slices and flip angle: the tilt of the magnetization for the longitudinal direction at the end of a radio frequency pulse

These segments were then assigned the appropriate tag color according to its tissue compartment location. Editing of the preliminary analysis was performed in the Region Growing/Paint mode. Each tag color was assigned a pixel minimum threshold that corresponded to its tissue. Corrections were made to regions that were incorrectly assigned a tag color. VAT and SAAT volume (millilitres) for each slice were determined by the following calculation: area of tag color (cm^2) x slice thickness (cm) / 1000. Total VAT (TVAT) and total SAAT (TSAAT) volume (litres) were determined by summing the individual slice volumes and dividing by 10^6. A more detailed protocol description of the protocol is included in **Appendix D**.

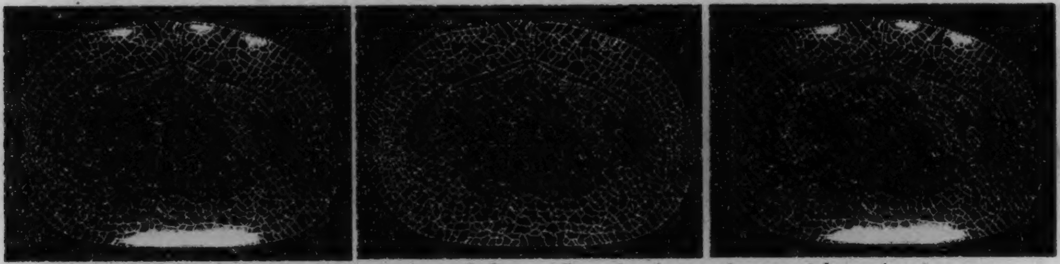

Figure 3-2 Mathematical morphology (left panel) and color assignment for subcutaneous abdominal adipose tissue (middle panel) and visceral adipose tissue (right panel).

3.2.6 Biochemical analysis

3.2.6.1 Insulin, glucose, lipids, CRP: Insulin, glucose, TC, TG, LDL-C, HDL-C and CRP concentrations were all measured at the UAH Laboratory (Capital Health, Edmonton, AB). Insulin concentrations were determined by semi-automated ECL (Elecysis 2010). Glucose was measured by a glucose oxidase method (LX 20, Beckman Coulter Instrumentation). The HOMA score was calculated using the following formula: fasting glucose (mmol/L) × fasting insulin (mU/mL) / 22.5 (Matthews *et al.*, 1985). Compared with the gold standard method (the euglycemic-hyperinsulinemic clamp), HOMA provides a valid estimate of IR. The original article on HOMA validity reported correlation coefficients as high as 0.92 in adult diabetic subjects and 0.83 in control studies (Matthews *et al.*, 1985) between HOMA and the euglycemic-hyperinsulinemic clamp method. More recent validation studies performed in the pediatric population have reported a correlation coefficient of 0.91 (Gungor *et al.*, 2004).

The validity of HOMA compared to the intravenous glucose tolerance test (IVGTT) is equally good (r = 0.50 for non-diabetic patients and r = 0.77 for diabetic patients) (Matthews *et al.,* 1985). Similarly, HOMA provides a reliable measure of IR. When measured on 2 separate days, the coefficient of variation for HOMA was 40%; however, IR estimates from a mean HOMA score obtained at 0, 5 and 10 minutes correlated strongly ($r = 0.98$, $p < 0.05$) with HOMA scores obtained every minute for 15 consecutive minutes. Fasting TC, TG, LDL-C and HDL-C concentrations were determined using a Roche Hitachi 917 (Roche, Laval, QC). TC was measured by the cholesterol oxidase method, TG was measured using a fully enzymatic colorimetric assay reaction for glycerol following the removal of all serum-free glycerol, and HDL-C was measured by photometry after the addition of polyethylene glycol-modified enzymes. LDL-C was calculated from the following formula: LDL-C (mmol/L) = TC (mmol/L) - HDL-C (mmol/L) x (TG (mmol/L) / 2.2) (Friedewald *et al.,* 1972). CRP was measured by immunoassay on the Synchron LX 20 Pro (Beckman Coulter, Mississauga, ON).

3.2.6.2 Apolipoprotein B48: Apo-B48 isolation and quantitation was performed according to the modified SDS-PAGE, immunoblotting and ECL method described by Smith *et al.* (1997) and Vine *et al.* (2007). Briefly, plasma samples and human standard (*d* < 1.006 g/mL) were mixed with a sample buffer / reducing agent (2-mercaptoethanol; Sigma-Aldrich, Oakville ON, catalogue number (CN) 60242, and NuPAGE LDS sample buffer; Invitrogen, Carisbad, CA, CN: NP007) to denature the proteins. Twenty-five microliters of prepared sample was loaded with a 20 µL micropipette (2 x 12.5 µL) into the wells (1.0 mm X 10) of a NUPAGE 3-8% Tris-Acetate gel (Invitrogen, Burlington ON, CN: EA0375BOX). The gel apparatus was assembled according to package instructions and electrophoresis occurred. Protein bands separated according to molecular weight. The separated protein bands were electrotransfered to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P transfer membranes, Millipore Corporation, Bedford, MA, CN: IPVH00010). Following transfer, the PVDF membrane was treated with double-distilled water and ethanol. After electrotransfer, the PVDF membrane was incubated overnight with a 2% Amersham protein blocking solution (ECL Advance blocking agent, Amersham / GE Healthcare UK Ltd., Little Chalfont Buckinghamshire and TBST) in a 4°C cold room. Following incubation, the PVDF membrane was treated with the primary antibody, an affinity purified goat polyclonal IgG antibody (Santa Cruz Biotech, Santa Cruz, CA, CN: sc11795) against a peptide on the amino terminus of apo B proteins. After a series of timed washes (55 minutes (min): 2 x 2 min; 2 x 3 min; 3 x 5 min; 2 x 10 min) with TBST, the PVDF membrane was incubated with a secondary antibody, the donkey anti-goat IgG-HRP anti-goat (Santa Cruz Biotech, Santa Cruz, CA, CN: sc2304). A final series of washes (115 mins: 2 x 2 min; 2 x 3 min; 3 x 5 min; 6 x 10 min; 2 x 15min) were performed before the apo-B48 bands were visualised using ECL (ECL-Advance, Amersham Biosciences, UK) by a Typhoon TRIO+ Variable Mode Imager (Amersham / GE Healthcare, Baie d'Urfé, QC). Apo-B48 bands were quantified by densiometry using the computer program *Image J* (1.37a, National Institutes of Health, USA) and compared to a purified apo-B48 standard of a known mass (16.91 µg/ml) to calculate the apo-B48 plasma concentration.

3.2.7 *Risk factor clustering*: Outcome variables were grouped and used to classify participants according to risk based on whether values were above / below accepted thresholds. Dyslipidemia, pre-diabetes, BP and adiposity were the outcome variables used to assign risk for several reasons: (1) both animal and human studies have identified these variables as important contributors to atherosclerosis, (2) although the definitions vary, many national and international public health groups (i.e., WHO, NCEP, International Diabetes Federation (IDF)) agree that these variables are key components of the metabolic syndrome which is linked to CVD (IDF, 2007), (3) these variables allowed for maximal data collection with minimal invasiveness or burden to the participants, which is particularly relevant when studying within a pediatric population, and (4) these factors were of greatest interest in terms of examining the relationship between apo-B48 concentrations and previously established CVD risk factors in children.

3.2.7.1 Dyslipidemia The National Cholesterol Education Program (NCEP) (NCEP, 1992) has published cut-off points for TG, TC, LDL-C and HDL-C to identify children and adolescents with abnormal lipid concentrations. Although not disease-related, to date, studies have used these cut-offs to separate children at increased risk of CVD from children at low risk of CVD. For children and adolescents (2-19 years old), the NCEP has three categories for lipid concentrations (normal, borderline and high / low). These categories correspond to the following percentiles: normal (< 75th percentile), borderline high (75th-95th percentile) and high (≥95th percentile) (**Table 3-2**) for TC, LDL-C and TG. For HDL-C the following definitions and thresholds are used: normal (> 25th percentile for HDL), borderline high (5th-25th percentile for HDL), and low (≤5th percentile). In each category, biochemical concentrations above the borderline cut-off point (or below for HDL-C) were considered abnormal. If the children had abnormal concentrations for all four lipid parameters (TC, TG, LDL-C and HDL-C) they were considered to have a dyslipidemic profile (**Table 3-3**).

Table 3-2 National Cholesterol Education Program classification criteria for TC, LDL-C, HDL-C and TG for children and adolescents aged 2-19 years old.

Lipid	Normal	Borderline High	High
TC (mmol/L)	< 4.40	4.40–5.18	≥5.18
LDL-C (mmol/L)	< 2.85	2.85–3.37	≥3.37
HDL-C (mmol/L)	> 1.56	1.55–0.91 (Borderline-low)	≤0.91 (Low)
TG (mmol/L)			
2–9 y	< 0.85	0.85–1.13	≥1.13
10–19 y	< 1.02	1.02–1.47	≥1.47

TC (total cholesterol); TG (triglyceride); HDL (high density lipoprotein); LDL (low density lipoprotein. Adapted from Joliffe *et al.* (2006).

3.2.7.2 Pre-diabetes: As shown in **Table 3-3,** IR and IGT, were included to provide a measure of risk of diabetes. Insulin resistance was measured using the HOMA calculated from the following formula: fasting glucose (mmol/L) × fasting insulin (μU/mL) /22.5 (Matthews *et al.,* 1985). A HOMA score ≥ 2 has been used to identify adults with IR, but it may overestimate its prevalence in children and adolescents (Keskin *et al.*, 2005). However, Keskin *et al.* (2005) calculated a HOMA cut-off point to diagnose IR in a sample of overweight (BMI $\geq$ 95th percentile) children and adolescents using a receiver operating characteristic scatter plot. This is advantageous because it provides a pediatric population-specific cut-off point for IR. Therefore, we used Keskin *et al'*s cut-off, HOMA > 3.16 to identify children with IR. Glucose tolerance is a measure of the hyperbolic relationship between insulin resistance and insulin secretion (Kahn *et al.,* 1993). Children with impaired glucose tolerance are insulin resistant, yet children with IR may not have IGT, particularly if their insulin secretion is adequately compensating for IR. Failure of the pancreatic β-cells to entirely compensate for increasing IR results in IGT, and if pancreatic insulin production remains inadequate, T2DM may develop (Chiasson & Rabasa-Lhoret, 2004). The established, 2-hour glucose concentration cut-off range (7.8 - 11.0 mmol/L) was used to identify children with IGT and is defined as a state of pre-diabetes (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2003).

3.7.2.3 Blood pressure: Cut-offs for high SBP and DBP in children were based on gender-, age-, and height- percentile tables (NHLBI, 2004). SBP and DBP were categorized separately into percentile groups and the higher of the two represented the overall BP percentile. Participants in this study with a BP ≥ 95th percentile were considered to have high blood pressure[8] (**Table 3-3**).

3.7.2.4 Adiposity: Clinically, a BMI ≥ 40 kg/m^2 is used a guideline to select severely obese adolescents for bariatric surgery, however there is no analogous cut-off point to define severe obesity in children (Freedman *et al.*, 2007). Similarly, there is no established definition of severe obesity using %BF for children. In our study, participants were stratified according to sample-derived cut-offs for BMI-z score and %BF. We used a cut-off point for BMI Z-score corresponding to the 97.5th percentile (Z-score ≥ 1.96) and the median %BF (≥ 43.9%) (**Table 3-3**).

3.7.2.5 Moderate versus high risk: Based on their dyslipidemic profile, pre-diabetic status, blood pressure, and adiposity, participants were classified as either *moderate risk* or *high risk* for CVD (**Table 3-4**). Children at moderate risk for CVD had 0 – 2 risk factors while children at high risk had 3 – 4 risk factors (**Table 3-4**). Several methods could have been used to classify risk group. It is important to note that a low risk group was not selected because it was hypothesized that based on the inclusion / exclusion criteria, all the subjects would have some degree of risk for CVD. Further, the moderate and high risk groups were selected because (1) previous studies defined participants with 3, 4 or 5 risk factors as belonging to a high risk group (Berenson *et al.,* 1998; Freedman *et al.,* 1999), (2) these categories would allow for straightforward, intra-sample comparisons, and (3) more statistical power could be achieved by limiting the number of groups used in analysis.

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[8] It is important to note that elevated blood pressure is distinct from hypertension. In this study we were unable to diagnose hypertension which is defined as consecutive measurements of elevated blood pressure, measure at three separate doctor's visits.

3.2.8 Statistical Analysis

All statistical analyses were performed using SPSS (version 14.0, 2005, Chicago, IL). Data symmetry was examined visually using histograms. Normality was determined using the Kolmogorov-Smirnov test for normality. Data distribution was not significantly different from normal, so variables were included in the analysis without transformation. If the sample size exceeded 4, within each cell, Chi-squared tests were used to compare categorical data, such as ethnicity and gender distribution. In order to determine if the participants in the current study had high apo-B48 concentrations we compared our results to the apo-B48 concentrations, reported in published studies that used a similar SDS-PAGE / immunoblotting technique.

Table 3-3 Classification criteria for risk factor clustering analysis.

CVD Risk Factor		Cut-off Points	
Dyslipidemia	TG (2-9 year olds)	Acceptable Borderline or high	< 0.85 mmol/L ≥0.85 mmol/L
	(10-19 year olds)	Acceptable Borderline or high	< 1.02 mmol/L ≥1.02 mmol/L
	TC	Acceptable Borderline or High	< 4.40 mmol/L ≥4.40 mmol/L
	LDL-C	Acceptable Borderline or High	< 2.85 mmol/L ≥2.85 mmol/L
	HDL-C	Acceptable Borderline or Low	> 1.66 mmol/L ≤1.55 mmol/L
Pre-diabetes	HOMA	Not IR IR	< 3.16 ≥3.16
	2-hr glucose	NGT IGT	< 7.8 mmol/L 7.8-11.1 mmol/L
Blood pressure	BP percentile	Acceptable High	< 95th percentile ≥95th percentile
Adiposity	BMI Z-score	Obese Severely obese	< 1.96 ≥1.96
	%BF	Obese Severely obese	< 43.5% ≥43.5%

TC (total cholesterol); TG (triglyceride); HDL-C (high density lipoprotein); LDL –C (low density lipoprotein, HOMA (homeostasis model assessment of insulin resistance), hr (hour), NGT (normal glucose tolerance), IR (insulin resistant), IGT (impaired glucose tolerance), BP (blood pressure), BMI (body mass index) %BF (percent body fat)

Published results were compared to the 95% CI derived from the current study's mean apo-B48 concentrations. In addition, one sample independent t-distribution tests were used to compare sample mean concentrations to published literature concentrations. To examine the relationship between apo-B48 and established CVD risk factors, a number of statistical tests were performed. Relationships between parameters were evaluated using Pearson correlations. When examining differences between boys and girls, two-sample independent t-tests were performed. In order to determine the influence of gender and ethnicity, both were entered as covariates in partial correlation analysis. For comparison between risk factor groups, the sample size was less than 10 and the distribution of data within these groupings was no longer normal, so non-parametric tests were used. A stepwise, multivariate linear regression model was used to examine significant predictor of apo-B48 concentration. Similarly, a stepwise multivariate logistic regression model was used to determine the contribution that apo-B48 concentration made to predicting risk group classification. For both models, all variables with a p-value < 0.25 from the correlation matrix (Hosmer & Lemeshow, 2000) were entered into the regression models. Subjects with missing data were excluded from the statistical tests on a test-by-test basis. All data were reported as mean ± standard deviation (SD) unless otherwise indicated. A p-value < 0.05 was considered to be significant for all analysis.

Table 3-4 Classification criteria for CVD risk groups[9].

Risk group	Number of CVD risk factors
Moderate risk	0 1 2
High risk	3 4

[9] Risk factors include dyslipidemia, pre-diabetes, blood pressure, and adiposity. Moderate risk refers to 0-2 risk factors and high risk refers to 3-4 risk factors.

Chapter 4: Results

4.1 Subject Characteristics

4.1.1 Demographic: **Table 4-1** shows the characteristics of the forty children (19 girls and 21 boys) who participated in the study. The mean age (± standard deviation (SD)) was 9.9 ± 1.6 years old. The majority of girls (78.9%) and boys (81.0%) were Caucasian. There were no significant differences in ethnicity distribution between genders, ($X^2 = 0.13$ for Caucasians and $X^2 < 0.01$ for Non-Caucasians). Half the non-Caucasian boys (n = 2) were Asian and the other half (n = 2) were of mixed ethnicity or "other". Hispanic, Asian, African, and Aboriginal individuals made up the non-Caucasian cohort of girls.

Table 4-1 Demographic characteristics.

Characteristics		Total (n = 40)	Girls (n = 19)	Boys (n = 21)	p-value
Age (years)		9.9 ± 1.6[11] (6.0-11.9)	9.8 ± 2.5 (6.4-11.2)	10.6 ± 1.8 (6.0-11.9)	0.54
Ethnicity					
Caucasian		2 (80.0)[12]	15 (78.9)	17 (81.0)	
Non-Caucasian		8 (20.0)	4 (21.1)	4 (19.0)	-
	Hispanic	1 (2.5)	1 (5.3)	0 (0.0)	
	Asian	1 (2.5)	0 (0.0)	2 (9.5)	-
	African	1 (2.5)	1 (5.3)	0 (0.0)	-
	Aboriginal	2 (5.0)	2 (10.5)	0 (0.0)	-
	Other[13]	2 (5.0)	0 (0.0)	2 (9.5)	

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[11] Mean ± SD (range)

[12] Mean (percent)

[13] "Other" refers to individuals that reported belonging to two different ethnic groups.

4.1.2 Anthropometry: The anthropometric characteristics are summarized in **Table 4-2**. No significant gender differences were found between boys and girls. All subjects had a WC $\geq 90^{th}$ percentile. In addition, across all variables of interest, there was no statistically significant difference in variance between boys and girls (all $p > 0.05$).

4.1.3 Biochemical: Biochemical characteristics are detailed in **Table 4-3**. Again, there were no significant differences between boys and girls. There was a trend towards greater CRP and apo-B48 concentrations in boys; however, neither difference achieved statistical significance ($p = 0.07$ and $p = 0.10$, respectively). Partial correlation analysis (data not shown) indicated that ethnicity did not significantly influence biochemical characteristics with the exception of DBP. Non-Caucasian girls had higher DBP compared to Caucasian girls (75.8 *versus* 66.2 mmHg; $p = 0.04$). In addition, across all variables of interest, there was no statistically significant difference in variance between boys and girls (all $p > 0.05$).

Table 4-2 Anthropometric and body composition characteristics

Characteristics	Total (n = 40)	Girls (n = 19)	Boys (n = 21)	p-value
Height (cm)	143.7 ± 10.7 (113.3-165.1)	143.6 ± 8.2 (124.1-160.0)	144.5 ± 12.7 (113.3-165.1)	0.79
Weight (kg)	57.2 ± 13.1 (33.4-91.4)	55.9 ± 11.6 (39.2-89.0)	59.1 ± 14.4 (33.4-91.4)	0.43
BMI (kg/m^2)	27.4 ± 3.2 (21.7-34.7)	27.2 ± 3.4 (23.0-34.7)	28.0 ± 3.3 (21.7-34.5)	0.30
BMI percentile	98.2 ± 1.4 (94.2-99.9)	97.9 ± 1.3 (95.0-99.7)	98.4 ± 1.5 (94.2-99.9)	0.25
BMI Z-score	2.2 ± 0.4 (1.6-3.1)	2.1 ± 0.3 (1.7-2.7)	2.3 ± 0.4 (1.6-3.1)	0.12
WC (cm)	92.3 ± 9.5 (75.3-117.4)	90.3 ± 8.7 (78.6-110.5)	94.1 ± 10.0 (75.3-117.4)	0.82
FM (kg)	24.3 ± 7.8 (12.8-46.3)	24.1 ± 6.3 (15.4-41.6)	24.9 ± 9.3 (12.8-46.3)	0.76
LTM (kg)	30.0 ± 5.7 (18.9-44.1)	28.9 ± 5.5 (20.9-44.1)	31.2 ± 5.8 (18.9-41.2)	0.21
%BF (%)	44.1 ± 4.8 (33.4-54.1)	45.1 ± 2.8 (40.2-49.6)	43.4 ± 6.2 (33.4-54.1)	0.28
% BL (%)	55.9 ± 4.9 (45.9-66.6)	54.8 ± 3.0 (50.3-59.8)	56.6 ± 6.2 (45.9-66.6)	0.25
TFM (kg)	12.5 ± 5.2 (5.4-31.7)	12.0 ± 3.6 (7.3-21.9)	13.1 ± 6.4 (5.4-31.7)	0.49
TLM (kg)	13.6 ± 2.6 (8.6-19.7)	13.0 ± 2.6 (9.5-19.7)	14.1 ± 2.5 (8.6-18.5)	0.16
UVAT (mL) (n = 39)	44.9 ± 14.1 (2.4-7.5)	42.1 ± 12.7 (2.6-7.5)	47.0 ± 15.2 (2.4-7.3)	0.28
TVAT (L) (n = 35)	2.0 ± 0.7 (0.9-3.7)	1.9 ± 0.8 (0.9-3.7)	2.1 ± 0.6 (1.1-3.5)	0.59
USAAT (mL) (n = 39)	206.2 ± 60.0 (117.3-399.7)	204.7 ± 44.3 (117.3-296.9)	211.5 ± 73.1 (125.8-399.7)	0.73
TSAAT (L) (n = 35)	8.0 ± 2.8 (4.2-17.1)	8.1 ± 2.1 (4.7-12.8)	8.1 ± 3.3 (4.2-17.1)	0.92

Data are expressed as mean ± SD and (range)

Abbreviations: n (number), BMI (body mass index), WC (waist circumference), FM (fat mass), LTM (lean tissue mass), %BF (body fat), % BL (body lean), TFM (trunk fat mass), TLM (trunk lean mass), UVAT (umbilical visceral adipose tissue), TVAT (total visceral adipose tissue), USAAT (umbilical subcutaneous abdominal adipose tissue), TSAAT (total subcutaneous abdominal adipose tissue)

Table 4-3 Blood pressure and biochemical characteristics of the 40 children.

Characteristics	Total (n = 40)	Girls (n = 19)	Boys (n = 21)	p-value
SBP (mmHg)	106.8 ± 10.4 (87.3-122.0)	107.7 ± 10.4 (87.3-122.0)	106.9 ± 10.1 (90.7-121.3)	0.82
DBP (mmHg)	67.8 ± 9.5 (50.0-85.3)	68.2 ± 9.4 (53.3-85.3)	68.3 ± 9.0 (50.0-81.3)	0.97
0-hr glucose (mmol/L)	4.7 ± 0.3 (4.1-5.7)	4.6 ± 0.3 (4.1-5.0)	4.7 ± 0.3 (4.2-5.7)	0.24
2-hr glucose (mmol/L)	6.0 ± 1.0 (4.0-8.4)	5.7 ± 1.0 (4.0-7.7)	6.1 ± 1.0 (4.5-8.4)	0.18
Fasting insulin (mU/L)	15.8 ± 7.8 (1.0-37.4)	17.2 ± 6.9 (10.6-36.0)	14.7 ± 8.7 (1.0-37.4)	0.36
HOMA	3.3 ± 1.8 (0.2-9.5)	3.5 ± 1.4 (2.3-7.7)	3.2 ± 2.1 (0.2-9.5)	0.55
CRP (mg/L)	3.1 ± 2.4 (0.2-9.0)	2.2 ± 1.9 (0.2-6.1)	3.7 ± 2.6 (0.2-9.0)	0.07
Fasting TC (mmol/L)	4.3 ± 0.8 (2.3-5.7)	4.3 ± 0.7 (2.6-5.3)	4.2 ± 0.9 (2.3-5.7)	0.85
Fasting TG (mmol/L)	1.2 ± 0.1 (0.4-3.1)	1.1 ± 0.5 (0.4-1.7)	1.2 ± 0.8 (0.4-3.1)	0.63
Fasting HDL-C (mmol/L)	1.0 ± 0.2 (0.7-1.7)	1.1 ± 0.2 (0.7-1.7)	1.0 ± 0.2 (0.7-1.7)	0.62
Fasting LDL-C (mmol/L)	2.6 ± 0.7 (1.2-3.9)	2.7 ± 0.6 (1.2-3.6)	2.6 ± 0.7 (1.4-3.9)	0.65
Fasting Apo-B48 (mg/L)	23.5 ± 6.7 (8.9-37.9)	21.5 ± 6.7 (8.9-36.1)	25.1 ± 6.7 (12.2-37.9)	0.10

Data are expressed as mean ± SD and (range)
Abbreviations: SBP (systolic blood pressure), DBP (diastolic blood pressure), hr (hour), HOMA (homeostasis model assessment of insulin resistance), CRP (C reactive protein), TC (total cholesterol), TG (triglyceride), HDL-C (high density lipoprotein), LDL-C (low density lipoprotein), Apo-B48 (apolipoprotein-B48)

4.2 Apolipoprotein-B48

4.2.1 *Comparison to adult data*: The mean apo-B48 concentration was higher in the pre-pubertal sample compared to concentrations reported previously in normal healthy, non-obese adults (Chan *et al.*, 2002a; Dane-Stewart *et al.*, 2001;

Dane-Stewart *et al.*, 2003; Mamo *et al.*, 2001; Redgrave *et al.*, 2001; Smith *et al.*, 1999). The mean apo-B48 concentration for non-obese subjects in each of the previously published studies fell outside the 95% CI for the sample mean apo-B48 concentration (21.2 - 25.7 mg/L). In comparison to obese subjects from previous reports, the mean pediatric apo-B48 concentration in the current study was significantly higher ($p < 0.01$) than values reported in a sample of overweight women with diabetes (Dane-Stewart *et al.*, 2003), similar to obese hyperinsulinemic men (Chan *et al.*, 2002a), and significantly lower than the normal healthy obese men (Mamo *et al.*, 2001) (**Table 4-4**).

Table 4-4 Comparison of sample mean apo-B48 concentration to means in published reports that used the SDS-PAGE, immunoblotting technique to quantify apo-B48

Reference	Subjects	Apo-B48 Concentration (mg/L)		p-value
		Adult	Pre-pubertal children (current study)	
(Dane-Stewart *et al.*, 2003)	Non-diabetic	13.0 (0.92)[14]	23.5 ± 1.1	<0.01
	Diabetic	16.4 (1.2)		<0.01
(Chan *et al.*, 2002a)	Non-obese	12.3 ± 2.8[15]		<0.01
	Obese	24.3 ± 8.8		0.45
(Dane-Stewart *et al.*, 2001)	FH controls	12.8 [7.3 – 28.6][16]		<0.01
(Mamo *et al.*, 2001)	Non-obese	12.7 (1.7)		<0.01
	Obese	31.5 (7.5)		<0.01
(Redgrave *et al.*, 2001)	Non-obese	6.9 (1.1)		<0.01
(Smith *et al.*, 1999)	Non-obese	6.9 (1.1)		<0.01

[12] mean (standard error of the mean

[13] mean ± SD

[14] mean [range]

Abbreviations: FH (familial hypercholesterolemia)

4.2.2 Correlations: As shown in **Table 4-5**, apo-B48 was not significantly related to any of the anthropometric and body composition measures (all $p > 0.05$). **Table 4-6** shows that the relationship between TG and apo-B48 was the only one to reach statistical significance ($r = 0.50$; $p = 0.01$). Apo-B48 was not significantly related to any other lipid sub-fractions, glucose / insulin measures, or BP (all $p > 0.05$). **Tables 4-7** and **4-8** display Pearson correlations for girls and boys separately. In boys, but not in girls, apo-B48 was positively correlated with fasting insulin, HOMA, TC, and TG.

Table 4-5 Pearson correlation matrix for apo-B48 and anthropometric and body composition variables (total sample)

Characteristics	Apo-B48	Height	Weight	BMI	BMI %ile	BMI Z-score	WC	FM	LTM	%BF	%BL	TFM	TLM	UVAT	TVAT	TSAAT	TSAAT
Apo-B48	-	0.13	0.22	0.27	0.20	0.06	0.27	0.18	0.23	0.05	-0.03	0.04	0.27	0.26	0.27	0.07	0.08
Height	0.13	-	0.84**	0.36**	-0.25	-0.43**	0.69**	0.72**	0.87**	0.26	-0.26	0.61**	0.80**	0.35**	0.51**	0.63**	0.64**
Weight	0.22	0.84**	-	0.81**	0.26	0.10	0.91**	0.96**	0.91**	0.58**	-0.56**	0.84**	0.86**	0.62**	0.79**	0.85**	0.91**
BMI	0.27	0.36**	0.81**	-	0.71**	0.60**	0.83**	0.85**	0.63**	0.70**	-0.69**	0.78**	0.63**	0.69**	0.79**	0.76**	0.85**
BMI %ile	0.20	-0.25**	0.26	0.71**	-	0.92**	0.37*	0.37*	0.10	0.48**	-0.47**	0.40*	0.17	0.51**	0.58**	0.35**	0.53**
BMI Z-score	0.06	-0.43**	0.10	0.60**	0.92**	-	0.21	0.22	-0.05	0.36*	-0.34*	0.26	0.01	0.40*	0.51**	0.24	0.50**
WC	0.27	0.69**	0.91**	0.83**	0.37*	0.21	-	0.91**	0.79**	0.62**	-0.61**	0.77**	0.79**	0.59**	0.80**	0.90**	0.88**
FM	0.18	0.72**	0.96**	0.85**	0.37*	0.22	0.91**	-	0.76**	0.77**	-0.76**	0.87**	0.71**	0.56**	0.75**	0.91**	0.95**
LTM	0.23	0.87**	0.91**	0.63**	0.10	-0.05	0.79**	0.76**	-	0.21	-0.19	0.68**	0.97**	0.64**	0.71**	0.62**	0.68**
%BF	0.05	0.26	0.58**	0.70**	0.48**	0.36*	0.62**	0.77**	0.21	-	-1.00**	0.66**	0.17	0.28	0.51**	0.76**	0.77**
%BL	-0.03	-0.26	-0.56**	-0.69**	-0.47**	-0.34*	-0.61**	-0.76**	-0.19	-1.00**	-	-0.65**	-0.15	-0.26	-0.49**	-0.75**	-0.76**
TFM	0.04	0.61**	0.84**	0.78**	0.408	0.26	0.77**	0.87**	0.68**	0.66**	-0.65**	-	0.63**	0.59**	0.70**	0.73**	0.77**
TLM	0.27	0.80**	0.86**	0.63**	0.17	0.01	0.79**	0.71**	0.97**	0.17	-0.15	0.63**	-	0.70**	0.75**	0.59**	0.64**
UVAT	0.26	0.35*	0.62**	0.69**	0.51**	0.40	0.59**	0.56**	0.64**	0.28	-0.26	0.59**	0.70**	-	0.85**	0.41**	0.52**
TVAT	0.27	0.51**	0.79**	0.79**	0.58**	0.51**	0.80**	0.75**	0.71**	0.51**	-0.49**	0.70**	0.75**	0.85**	-	0.65**	0.73**
USAT	0.07	0.63**	0.85**	0.76**	0.35*	0.24	0.90**	0.91**	0.62**	0.76**	-0.75**	0.73**	0.59**	0.41**	0.65**	-	0.95**
TSAT	0.08	0.64**	0.91**	0.85**	0.53**	0.50**	0.88**	0.95**	0.68**	0.77**	-0.76**	0.77**	0.64**	0.52**	0.73**	0.95**	-

*p < 0.05; **p< 0.01

Abbreviations: Apo-B48 (apolipoprotein-B48), BMI (body mass index), BMI %ile (BMI percentile), WC (waist circumference), FM (fat mass), LTM (lean tissue mass), %BF (percent body fat), %BL (percent body lean tissue), TFM (trunk fat mass), TLM (trunk lean mass), UVAT (umbilical visceral adipose tissue), TVAT (total visceral adipose tissue), USAAT (umbilical visceral abdominal adipose tissue), TSAAT (total visceral abdominal adipose tissue)

Table 4-6 Pearson correlation matrix for apo-B48, blood pressure and biochemical variables (total sample)

	Apo-B48	SBP	DBP	0-hr glucose	2-hr glucose	0-hr insulin	HOMA	CRP	TC	TG	HDL-C	LDL-C
Apo-B48	-	0.08	-0.14	0.24	0.04	0.14	0.17	0.26	0.31	0.50**	-0.12	0.18
SBP	0.08	-	0.74**	-0.12	0.10	0.40*	0.36*	0.28	0.28	0.20	0.05	0.23
DBP	-0.14	0.74**	-	0.00	0.09	0.25	0.24	0.09	0.12	-0.08	0.03	0.15
0-hr glucose	0.24	-0.12	0.00	-	0.20	0.37*	0.49**	0.03	-0.01	-0.01	-0.32*	0.09
2-hr glucose	0.04	0.10	0.09	0.20	-	0.30	0.32*	0.33	0.47**	0.14	0.08	0.44**
0-hr insulin	0.14	0.40*	0.25	0.37*	0.30	-	0.99**	0.46**	0.21	0.24	-0.24	0.19
HOMA	0.17	0.36*	0.24	0.49**	0.32*	0.99**	-	0.45*	0.20	0.21	-0.26	0.21
CRP	0.26	0.28	0.09	0.03	0.33	0.46**	0.45*	-	0.24	0.31	-0.22	0.21
TC	0.31	0.28	0.12	-0.01	0.47**	0.21	0.20	0.24	-	0.30	0.31	0.91**
TG	0.50**	0.20	-0.08	-0.01	0.14	0.24	0.21	0.31	0.30	-	-0.37*	0.02
HDL-C	-0.12	0.05	0.03	-0.32*	0.08	-0.24	-0.26	-0.22	0.31	-0.37*	-	0.20
LDL-C	0.18	0.23	0.15	0.09	0.44**	0.19	0.21	0.21	0.91**	0.02	0.20	-

*$p < 0.05$; **$p < 0.01$

Abbreviations: Apo-B48 (apolipoprotein-B48), SBP (systolic blood pressure), DBP (diastolic blood pressure), hr (hour), HOMA (homeostasis model assessment of insulin resistance), CRP (C-reactive protein), TC (total cholesterol), TG (triglyceride), HDL-C (high density lipoprotein), LDL-C (low density lipoprotein).

Table 4-7 Pearson correlation matrix for apo-B48, blood pressure and biochemical variables by gender

Gender	Variable	Height	Weight	BMI	BMI %ile	BMI Z-score	WC	FM	LTM	%BF	%BL	TFM	TLM	UVAT	TVAT	USAAT	TSAAT
Girl	Apo-B48	-0.13	0.11	0.29	0.32	0.37	0.07	0.05	0.19	-0.23	0.28	0.03	0.21	0.24	0.08	-0.15	0.02
Boy		0.26	0.26	0.18	0.03	-0.26	0.34	0.23	0.19	0.22	-0.22	0.00	0.23	0.23	0.45	0.17	0.10

*p < 0.05; **p < 0.01

Abbreviations: Apo-B48 (apolipoprotein-B48), BMI (body mass index), %ile (BMI percentile), WC (waist circumference), FM (fat mass), LTM (lean tissue mass), %bf (percent body fat), % BL (percent body lean tissue), TFM (trunk fat mass), TLM (trunk lean mass), UVAT (umbilical visceral adipose tissue), TVAT (total visceral adipose tissue), USAAT (umbilical subcutaneous abdominal adipose tissue), TSAT (total subcutaneous abdominal adipose tissue)

Table 4-8 Pearson correlation matrix for apo-B48, blood pressure and biochemical variables by gender

Gender	Variable	SBP	DBP	0-hr glucose	2-hr glucose	0-hr insulin	HOMA	CRP	TC	TG	HDL-C	LDL-C
Girl	Apo-B48	0.11	0.16	0.24	-0.25	-0.23	-0.20	0.32	0.02	0.46	-0.15	-0.08
Boy		0.08	-0.42	0.21	0.18	0.48*	0.45*	0.21	0.54*	0.55*	-0.06	0.41

*p < 0.05; **p < 0.01

Abbreviations: Apo-B48 (apolipoprotein-B48), SBP (systolic blood pressure), DBP (diastolic blood pressure), hr (hour), HOMA (homeostasis model assessment of insulin resistance), CRP (C-reactive protein), TC (total cholesterol), TG (triglyceride), HDL-C (high density lipoprotein), LDL-C (low density lipoprotein)

4.2.3 Predictors of apo-B48 concentration: Stepwise multivariate linear regression was conducted to determine if any of the parameters used in the Pearson correlation matrix predicted apo-B48 concentration. As described previously, all variables with a p-value < 0.25 (Hosmer & Lemeshow 2000) were entered into the model as well as age, gender and ethnicity (Caucasian and non-Caucasian). Neither age ($\beta = 0.15$, $p = 0.32$), ethnicity ($\beta = 0.03$, $p = 0.20$) nor gender ($\beta = 0.23$, $p = 0.11$) significantly influenced apo-B48 concentrations (**Table 4-9**). In the final model, TG accounted for approximately 26% of the variance ($R^2 = 0.26$; $p <$ a0.01) in apo-B48 concentration. No other variables contributed significantly to the model.

Table 4-9 Linear regression coefficients and p-values for all variables related to apo-B48 at $p < 0.25$

Variable	**Coefficient β**	**p-value**
TG	0.51	<0.01
Age	0.15	0.32
Gender	0.18	0.26
Ethnicity	0.03	0.20
BMI	0.13	0.43
UVAT	0.14	0.38
TVAT	0.14	0.40
LDL-C	0.21	0.18
TC	0.23	0.15
HOMA	0.07	0.67

Abbreviations: TG (triglyceride), BMI (body mass index), UVAT (umbilical visceral adipose tissue), TVAT (total visceral adipose tissue, TC (total cholesterol), HOMA (homeostasis model assessment of insulin resistance)

4.3 Risk factor prevalence and clustering

4.3.1 Prevalence of risk factors: According to NCEP guidelines (NCEP, 1992), all children had at least one lipid parameter above the borderline-high or borderline-low cut-off. Most had borderline high or high TC (56.4%), TG (53.8%), LDL-C (43.5%) and low HDL (97.4%). Insulin resistance was present in 43.6% of children and 5.3% of children had IGT. Elevated BP was the least common CVD risk factor (20.5%). Lastly, 69.2% and 48.7% of children were considered severely obese according to their BMI Z-score and %BF categories, respectively. (**Table 4-10**).

Table 4-10 Classification of normal and borderline-high-low groups according to dyslipidemia, pre-diabetes, blood pressure and adiposity

CVD Risk Factor		Cut-off Points		n (%)	G/B
Dyslipidemia	TC	Acceptable Borderline or High	< 4.40 ≥4.40	17(43.6) 22 (56.4)	8/9 10/12
	TG (2-9 yrs)	Acceptable Borderline or High	< 0.85 ≥0.85	8 (42.1) 10 (55.6)	3/5 7/3
	(10-19 yrs)	Acceptable Borderline or High	< 1.02 ≥1.02	10 (47.6) 11 (52.4)	5/5 3/8
	LDL-C	Acceptable Borderline or High	< 2.85 ≥2.85	22 (56.4) 17 (43.6)	9/13 9/8
	HDL-C	Acceptable Borderline or Low	> 1.66 ≤1.55	2 (5.3) 38 (94.7)	1/1 17/20
Pre-diabetes	IR: HOMA	Not IR IR	< 3.16 ≥3.16	22 (56.4) 17 (43.6)	9/13 9/8
	GT: 2-hr glucose	NGT IGT	< 7.8 mmol/L 7.8-11.1 mmol/L	36 (94.7) 2 (5.3)	17/19 0/2
BP	BP %ile	Acceptable High	< 95th percentile ≥95th percentile	31 (79.5) 8 (20.5)	15/17 4/4
Adiposity	Z-score	Obese Severely obese	< 1.96 ≥1.96	12 (30.8) 27 (69.2)	8/4 11/17
	%BF	Obese Severely obese	< 43.9% ≥43.9%	20 (51.3) 19 (48.7)	7/13 12/8

Abbreviations: n (number), TC (total cholesterol), TG (triglyceride), yrs (years), LDL-C (low density lipoprotein cholesterol), HDL-C (high density lipoprotein cholesterol), IR (insulin resistance), HOMA (homeostasis model assessment of insulin resistance), GT (glucose tolerance), hr (hour), NGT (normal glucose tolerance), IGT (impaired glucose tolerance), BP (blood pressure), %ile (percentile), %BF (percent body fat)

4.3.2 Moderate risk group versus high risk group: When divided into moderate and high risk factor groups, 87.2% (n = 34) were considered to be at moderate risk for CVD and 12.8% (n = 5) were considered to be at high risk for CVD (see **Table 4-11**). As shown in **Table 4-12**, all measures of anthropometry, except BMI percentile (p = 0.12) and UVAT (p = 0.06), were significantly different between moderate and high risk groups.

Table 4-11 Risk factor clustering into moderate or high risk groups based on number of risk factors

	Number of CVD risk factors	**n (%)**	**Girls/Boys**
Moderate risk	0	12 (30.8)	5/7
	1	11 (28.2)	5/6
	2	11 (28.2)	5/6
Total		**34 (87.2)**	**15/19**
High risk	3	4 (10.3)	2/2
	4	1 (2.6)	1/0
Total		**5 (12.8)**	**3/2**

Abbreviations: CVD (cardiovascular disease), n (number)

4.3.3 Apo-B48 as a predictor of risk group: The variance in apo-B48 between moderate and high risk groups was not significant (p = 0.07). All of the factors that differed between groups with a p-value < 0.25 in addition to apo-B48, gender, age and ethnicity were entered into a stepwise multivariate logistic regression model, to determine whether apo-B48 contributed to predicting risk group membership (**Table 4-13**). All measures of dyslipidemia, pre-diabetes, BP, %BF and BMI Z-score were excluded because they were used to originally determine risk group designation. In the final model, body weight emerged as the independent predictor of risk group, accounting for 55% of the variance (r^2 = 0.55; $p < 0.01$).

Table 4-12 Anthropometric and biochemical characteristics of the moderate risk group compared to the high risk group.

Characteristic	Moderate risk group[16] (n = 34)	High risk group (n = 5)	p-value[17]
Age (yrs)	9.9 ± 1.6	10.5 ± 1.0	0.43
Height (cm)	142.5 ± 10.3	153.7 ± 9.4	0.02
Weight (kg)	54.8 ± 10.9	74.7± 15.8	0.01
BMI (kg/m^2)	26.8 ± 2.9	31.3 ± 3.4	0.01
BMI %ile	98.0 ± 1.5	99.1± 0.5	0.12
WC (cm)	90.8 ± 8.6	102.3 ± 11.0	0.05
FM (kg)	22.8 ± 6.5	34.8 ± 9.5	0.01
LTM (kg)	29.0 ± 4.9	37.3 ± 6.4	0.02
TFM (kg)	11.7 ± 4.9	18.2 ± 5.0	0.00
TLM (kg)	13.1 ± 2.3	16.9 ± 2.9	0.01
UVAT (mL) (n = 33/5)	42.1 ± 13.2	58.1 ± 18.6	0.06
TVAT (L) (n = 29/5)	1.9 ± 0.6	2.8 ± 0.8	0.02
USAAT (mL) (n = 33/5)	197.9 ± 55.4	265.1 ± 68.0	0.04
TSAAT (L) (n = 29/5)	7.5 ± 2.5	11.3 ± 2.5	0.01
CRP (mg/L)	2.8 ± 2.1	5.5 ± 3.4	0.11
Apo-B48 (mg/L)	23.3 ± 7.3	24.8 ± 2.6	0.53

Abbreviations: yrs (years), BMI (body mass index), %ile (percentile), WC (waist circumference), FM (fat mass), LTM (lean tissue mass), TFM (trunk fat mass), TLM (trunk lean mass), UVAT (umbilical visceral adipose tissue), TVAT (total visceral adipose tissue), USAAT (umbilical subcutaneous abdominal adipose tissue), TSAAT (total subcutaneous abdominal adipose tissue), CRP (C reactive protein), Apo-B48 (apolipoprotein-B48).

[15] Risk groups for CVD (0 - 2 risk factor = moderate; 3 or 4 risk factors = high)

[16] p-value derived from Mann-Whitney U test (non-parametric)

Table 4-13 Logistic regression coefficients for all variables related to risk group with a $p < 0.25$ and apolipoprotein-B48

Variable	Coefficient β	p-value
Weight	0.51	0.00
Age	-0.21	0.27
Gender	-0.25	0.10
Ethnicity	-0.03	0.86
Height	-0.12	0.64
WC	-0.37	0.33
FM	0.20	0.71
LTM	-0.07	0.79
TFM	0.23	0.42
TLM	0.21	0.54
UVAT	0.06	0.77
TVAT	0.13	0.62
USAAT	-0.21	0.49
TSAAT	0.16	0.68
Apo-B48	0.01	0.15

Abbreviations: WC (waist circumference), FM (fat mass), LTM (lean tissue mass), TFM (trunk fat mass), TLM (trunk lean mass), UVAT (umbilical visceral adipose tissue), TVAT (total visceral adipose tissue, USAST (umbilical subcutaneous abdominal adipose tissue), TSAAT (total subcutaneous abdominal adipose tissue), Apo-B48 (apolipoprotein-B48)

Chapter 5: Discussion

5.1 General Discussion

5.1.1 Introduction: In this sample of overweight, pre-pubertal children apo-B48 was elevated compared to published reports of normal weight (BMI $\leq$25 kg/m^2) and overweight (BMI = 25-30 kg/m^2) adults. Triglyceride concentration was positively correlated with apo-B48 and independently predicted apo-B48 levels. Finally, apo-B48 concentration was not significantly different between moderate risk and high risk groups and did not predict CVD risk.

5.1.2 Comparison of apo-B48 to adult data: The results of this study support part 1 of our initial hypothesis; apo-B48 was elevated in this sample of overweight, pre-pubertal children compared to published adult data. In comparison to adult data for non-obese and non-diabetic individuals, our sample had higher apo-B48 concentrations. In addition, the apo-B48 levels of this sample were comparable to obese, insulin resistant adults. Since our report is the first to examine apo-B48 in children, there are no pediatric data to guide our analyses. As a result, these findings provide greater rationale for further study of chylomicrons in the pediatric population, particularly in normal weight children. If apo-B48 concentrations are found to be low in normal weight children and elevated in overweight children, the data would suggest that there is a threshold BMI where children are more likely to have elevated apo-B48 and perhaps at greater risk of CVD. Using a longitudinal study design, it would be important to determine whether elevations in apo-B48 precede the development of other CVD risk factors including abo-B48, to identify whether this measure could be used as an early measure of CVD risk.

5.1.3 Predictors of apo-B48 concentration: Triglyceride was the only predictor of apo-B48 in this study. Similar associations between TG and apo-B48 have been shown repeatedly in the literature in adults (Bjorkegren *et al.*, 2002; Chan *et al.*, 2002a; Cohn *et al.*, 1999; Couillard *et al.*, 2002; Marcoux *et al.*, 2000; Smith *et al.*, 1999). Triglyceride concentration is closely associated with apo-B48 concentration because chylomicrons contain a large proportion of circulating plasma TG. Furthermore, abnormalities in TG concentration are symptomatic of dysregulation in either the hepatically-derived or intestinally-derived lipoprotein subfractions. Although some reports have suggested that TG or the TRL-fraction is an adequate surrogate measure of apo-B48 (Imke *et al.*, 2005), these two measures do not distinguish between hepatically- and intestinally-derived lipoproteins. For instance, a recent report examined the influence of insulin on intestinal production rates of apo-B48 containing lipoproteins *in vivo (*Duez *et al., 2006)*. Duez *et al.* conducted kinetic studies on 14 normoglycemic men and found that production rates were significantly higher ($p = 0.04$) in men with higher fasting insulin concentrations and higher HOMA scores. The same relationship was not observed for TRL-TG and TRL-apo B fractions indicating that distinctions must be made between general TRL fraction and apo-B48-containing lipoprotein fractions. Given that chylomicrons play a pivotal role in atherosclerosis, it is important that they are individually studied to determine the response of the outcomes to intervention and thus improve the physiological relevance of these methods.

5.1.4 Risk factor clustering: Contrary to our hypothesis, apo-B48 was not significantly different between moderate risk and high risk groups. This could be due to a number of reasons. First, it is possible that high apo-B48 concentrations, in children do not contribute further to the CVD risk profile; this is unlikely considering the growing body of cellular, animal and adult data supporting a role for apo-B48 in CVD. Second, in this study, children already at high risk for CVD, may not exhibit any further differences between the risk groups; however, differences may emerge in other pediatric populations (i.e., normal weight, pubertal). Third, the lack of difference between moderate and high risk groups could be due to inadequate sample size and insufficient variation among the kids within the study. Apo-B48 concentration was marginally higher in the high risk group and perhaps, with a larger sample, it would be statistically significant. Finally, the lack of difference between risk groups could be due the limitations in the criteria used to assign risk groups. For instance, the 95th percentile may not be the best BMI cut-off to define children at greatest risk for future disease. A study by Freedman *et al.* (2007) proposed that the 99th percentile may represent a more clinically relevant cut-off because of group heterogeneity among boys and girls with a BMI ≥95th percentile. A cross-sectional analysis was conducted on 6731 children from Bogalusa, Louisiana. The prevalence of multiple risk factors for CVD (>3; dyslipidemia, high fasting insulin and high BP) did not increase greatly between the 90th and 95th BMI percentile (6% and 7%; respectively), but was markedly increased at the 99th (33%). Further, excess adiposity was observed in only 65% of children with a BMI ≥95th percentile compared to 94% of children with a BMI ≥99th. Since the greatest predictor of risk group was body weight, inclusion of the 99th %ile BMI category to the "high risk group" or raising the Z-score cut-off to the 99th percentile may have helped to better divide subjects according to risk. In addition, the number of risk factors assigned to each group could have been distributed in several different ways (e.g., only participants with 0 risk factors could have been included in the moderate risk group). Alternatively, a third risk group ("low") could have been added given the number of children with "0" risk factors. A comparison of three risk groups (low, moderate and high) may have revealed differences in apo-B48 concentration, but our small sample size precluded this option. Further, the conservative criteria (i.e., dyslipidemia = all four lipid abnormalities) may have underestimated the number of children who were classified in the high risk group, thus leading to greater variability in the moderate risk group and less statistical power to detect differences.

In our study, four CVD risk variables (dyslipidemia, pre-diabetes, blood pressure and adiposity) were used to identify increased risk for CVD. For dyslipidemia, we used the NCEP criteria for borderline and high / low lipid concentrations. The most common form of dyslipidemia was borderline-low HDL-C (94.7%) which is much higher than what has been reported previously in other pediatric studies. This difference is likely attributable to the fact that many previous studies have used the NCEP "low" cut-off (< 0.91 mmol/L). At this cut-off, 25% of our sample had abnormal HDL-C which is similar to what has been found in the other pediatric studies (Boyd *et al.,* 2005, Reinehr *et al.,* 2005). Yet, even in studies using the borderline-low cut-off (Webber *et al.*, 1991) the prevalence of low HDL-C was significantly lower compared to our study. Perhaps this difference in HDL-C concentration is due to differences in sample population since lipid concentrations have been shown to vary with race (NCEP, 2002) and the Bogalusa subject population is 50% African American compared to 2.5% in our sample. From a public health perspective, use of the borderline versus high cut-off for HDL-C would change the number of children identified with dyslipidemia. NCEP cut-offs are based on the 75th and 95th (5th, 25th for HDL-C) percentiles. It has been suggested that the use of percentiles may not be the best indicator of lipid abnormalities since the link between lipid percentiles in children and future disease in adulthood is not well established. Recently, Joliffe and Janssen (2006) established gender and age-specific thresholds for lipoprotein levels in adolescents based on the NHANES study and lambda-sigma-mu growth regression curves. Unfortunately these regression curves have not yet been extended to the pre-pubertal population, therefore we were unable to use these disease-related cut- points in our study.

Similarly, a diagnosis of IR in children is limited by the absence of widely established cut-offs for HOMA. The adult cut-off (HOMA ≥ 2.0) is reported to overestimate the prevalence of IR in the pediatric population. In our sample, a HOMA cut-off score of 2.0 would classify 66.7% of children as insulin resistant. Identification of IR in our study was based upon a study by Keskin *et al.* (2005). A limitation of this study was that HOMA was not validated against a gold standard method (i.e., the euglycemic-hyperinsulinemic clamp). Further validation of the Keskin *et al.* cut-off would improve the reliability of using HOMA to define IR in the participants of this study.

The criteria used to identify children with IGT are well established and featured in the latest Canadian clinical practice guidelines for diabetes (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2003). Studies indicate that approximately 20-30% of overweight American youth have IGT (Goran *et al.*, 2004; Sinha *et al.*, 2002; Weiss *et al.*, 2005). However, only 5.4% of the children in our sample had IGT. Previous published reports included a greater proportion of non-Caucasians and adolescents who are more likely to exhibit IGT due to genetic variation in insulin sensitivity, greater excess adiposity and the influence of sex hormones on glucose metabolism, which would likely account for the differences between studies. HOMA and 2-hour glucose provide unique information about insulin-glucose kinetics and no studies have examined the relative contribution of IR and IGT to hypersecretion or delayed clearance of chylomicrons. As such, it is unclear which measure would best relate to apo-B48 physiologically. This is important because there is mechanistic and kinetic evidence that hypersecretion of apo-B48 is related to hyperinsulinemia (Lewis *et al.*, 2005, Duez *et al.*, 2006). Without a measure of IR that is physiologically relevant to chylomicron metabolism, our data may falsely indicate no significant relationship between IR and apo-B48.

High BP cut-offs were determined using NHLBI guidelines (2004). Blood pressure is a good example of a CVD risk factor that has been well studied in both children and adults, which facilitates identification of those at risk. We were not able to determine whether the participants in the study had hypertension because BP was not measured on three separate occasions as per NHLBI guidelines. In future studies, identifying children with hypertension will better characterize risk factor groups.

We used both BMI Z-score and the median %BF to identify the most severely obese children and those at greatest risk of developing CVD. BMI Z-score is a useful tool to assess adiposity, because unlike percentiles Z-scores are a continuous variable, not bound from 0 - 100 like percentiles. Yet, because BMI Z-scores are normalized according to the skewed BMI population distribution, as the normal curve approaches infinity, the change in Z-score becomes smaller for an equivalent change in BMI. Consequently, the BMI Z-score scale is not consistent at extreme BMIs (Cole *et al.*, 2005). By contrast, %BF is not subject to statistical limitations. Also, %BF provides a more informative measure of body composition compared to BMI Z-score. Body composition testing using DXA is becoming more common because of its ease, speed and safety for the pediatric population. However, to date normalized values for %BF have not been published. A study in 4 to 11-year old boys and girls proposed using a 33% BF cut-off to define excess body fatness (Higgins *et al.*, 2002). Neovius *et al.* (2004) found that increasing the cut-off for females to 37.5% increased the sensitivity of identifying overweight girls. Freedman *et al.* (2005) also propose using the 85th and 95th percentile for %BF definitions, based on the sample population to identify overweight and severe obesity, respectively. Clearly, the debate is ongoing and it remains unclear what %BF confers the greatest CVD risk to children. In our study we used the median %BF to define those at high risk for CVD. The resultant 43.6% was higher than suggested in previous studies and may have led to an underestimation of children with severe obesity, however without established cut-offs, all percentages are arbitrary to some degree. In summary, in order to better assign risk groups and determine the relationship between CVD risk and apo-B48, more clinically-relevant cut-off points for dyslipidemia, insulin resistance and adiposity are needed.

5.1.5 Influence of gender: There were no significant differences in the anthropometric variables, body composition, BP or biochemical characteristics between boys and girls. The difference in apo-B48 concentrations approached significance; boys had higher fasting apo-B48 concentrations compared to girls (25.1 *versus* 21.5 mg/L, $p = 0.10$). The only other variable that neared significance was CRP; boys had higher concentrations than girls (3.7 *versus* 2.2, $p = 0.07$). Although partial correlation analysis (data not shown) confirmed that gender did not significantly influence the relationship between apo-B48 and other variables, when correlation analyses were performed with boys and girls individually, some differences emerged. For example, in our study, apo-B48 was significantly correlated (all $p < 0.05$) with fasting insulin ($r = 0.48$), HOMA ($r = 0.45$), TC ($r = 0.54$) and TG ($r = 0.55$) in boys alone. However, we did not observe any significant relationships in girls. Given that this is the first study to report apo-B48 in children, interpretation of this finding is challenging. It is possible that these gender differences are spurious and a larger sample would eliminate the differences. However, it is also possible that the preliminary data from this study indicates a potential relationship between gender and apo-B48.

It is well established that CVD risk (especially in adolescents and adults) is influenced by gender (Public Health Agency of Canada, 1997). For example, in all age groups until menopause, risk of CVD in women lags 10-15 years behind the equivalent risk in men (NCEP, 2002). By contrast, pediatric reports in children have been less consistent. Some studies have not observed gender differences (Freedman *et al.*, 2001; Reinehr *et al.*, 2005) while others have demonstrated that dyslipidemia can be influenced by gender during the pre-pubertal period. For example, boys have been shown to have higher TG, LDL-C and TC levels and lower HDL-C levels compared to girls (Chu *et al.*, 1998; Maffeis *et al.*, 2001). Despite reported differences in lipid concentrations between boys and girls, relationships between variables were always consistent. To date, there have been no published studies regarding the influence of gender on apo-B48 concentration, however, comparisons between studies which have included men and women only suggest no significant differences exist between men and women (Chan *et al.*, 2002a; Dane-Stewart *et al.*, 2003). Although the role of gender in CVD risk is not fully understood, sex hormones (i.e., estrogens and androgens) are thought to play an important role. Among its many roles, estrogen is responsible for a gynoid body fat partitioning pattern, increased glucose tolerance, decreased endothelial reactivity, increased blood pressure, reduced vascular inflammation and reduced LDL-C concentration lipid (Klouche, 2006; Rosano *et al.*, 2007). Yet, estrogen replacement therapy in post-menopausal women has had mixed success at reducing the incidence of CVD (Rossouw, 2006). In men, androgens may also have an important role since the prevalence of CVD increases with decreased testosterone levels (English *et al.*, 2000). While correlation analysis for each gender revealed some differences, overall gender was not a significant influence on apo-B48 concentrations or its relationship with other variables. The lack of statistically significant gender differences in our study may be attributed to the pre-pubertal status of the participants. Although estrogen concentrations were not measured in our sample, it is possible that its concentration was not significantly different between boys and girls, thus its influence could be minimized. In fact, the marked lack of difference between boys and girls in all anthropometric variables and body composition attests to the lack of visible influence of sex hormones on sexual maturation among study participants. However, sex hormones may influence lipid metabolism prior to visible sexual maturation. Additional testing to study changes in apo-B48 concentration throughout puberty and menopause would contribute valuable information to the influence of gender on chylomicron metabolism and risk of CVD.

5.2 Potential Confounding Factors

5.2.1 Ethnicity: It has also been widely reported that CVD risk varies with ethnicity (Herd *et al.*, 2001; Weiss *et al.*, 2004). For instance, African American children have been shown to have lower TG and higher LDL-C *versus* Caucasians (Herd *et al.*, 2001). Similarly, Asian children are reported to have lower HDL-C concentrations compared to their Caucasian counterparts (Dwyer *et al.*, 1997). Risk of IR and / or IGT also varies based on ethnicity. There is an increased prevalence of insulin resistance among American American (Weigensberg *et al.*, 2005) and Hispanic (Lee *et al.*, 2006) children compared to Caucasian children. Ethnicity also influences body fat distribution (Bacha *et al.*, 2003; Yanovski *et al.*, 1996) and hypertension (Cruickshank *et al.*, 2005; Dekkers *et al.*, 2002) with higher SBP and DBP levels reported in children of African descent compared to their Caucasian peers (Bacha *et al.*, 2003; NCEP, 1992). Since most studies have been performed exclusively on Caucasian adults, it is unknown how ethnicity influences apo-B48 concentration. We observed higher DBP among non-Caucasian girls, but other variables did not vary according to ethnicity. Apo-B48 concentrations did not differ significantly between Caucasians and non-Caucasians. Elevated apo-B48 concentrations could increase risk of CVD in various ethnic groups to different degrees. For instance, a given elevated apo-B48 concentration could confer greater risk to people of African descent, compared to their Caucasian counterparts because of the already accelerated atherogenesis in Blacks, mediated by hypertension. By contrast, a similarly elevated apo-B48 concentration may be less deleterious in Asian men and women who are at low risk of CVD. Certainly, ethnic variability would be an important factor to consider in future studies.

5.2.2 Anthropometry, body composition and apo-B48: Previous adult studies have reported a positive relationship between apo-B48 concentration and abdominal obesity (as measured by WC) in middle aged men (Chan *et al.*, 2002a; Couillard *et al.*, 2002; Mamo *et al.*, 2001). Despite the presence of a high degree of body fat in our sample (45% BF) and elevated apo-B48 concentrations, apo-B48 was not associated with any measure of body fatness. Previous pediatric studies have reported a significant relationship between total and / or central fat (measured by DXA) and TG, HDL-C (Daniels *et al.*, 1999; Teixeira *et al.*, 2001) and IR (Gower *et al.*, 1999). Abdominal VAT, measured by CT, has also been shown to be independently related to TG and fasting insulin (Gower *et al.*, 1999; Herd *et al.*, 2001) in pre-pubertal children. In our study, TG was not significantly related to any anthropometric measure or body composition (data not shown). Our data differ from these previous reports, but sample difference (i.e., ethnicity, adiposity and age) may account for this inconsistency.

5.2.3 Insulin and apo-B48: The literature relating apo-B48 to IR is equivocal. Animal studies indicate that IR leads to intestinal apo-B48 overproduction and can be reduced by decreasing IR (Haidari *et al.*, 2002; Lewis *et al.*, 2005). Insulin resistance may also indirectly mediate chylomicron particle accumulation by increasing its competition with hepatically-derived lipoproteins for lipolysis and clearance. Insulin increases hepatic fatty acid oxidation via a phosphorylation cascade, which in turn can decrease hepatic VLDL production. Insulin also suppresses mobilization of non-esterified fatty acids (NEFAs) from adipocyte stores via inhibitory phosphorylation of hormone sensitive lipase, thereby reducing NEFA flux to the liver and VLDL production. However, an 8-week intervention with metformin and rosiglitazone (two insulin sensitizing-drugs) which improved IR did not improve either fasting or postprandial chylomicron levels in obese-insulin resistant men (James *et al.*, 2005). Coupled with the fact that apo-B48 was not significantly related to HOMA or IGT in our study, the intervention studies suggest that elevated fasting apo-B48 may be mediated by a mechanism independent of insulin.

5.2.4 Adipocytokines and apo-B48: Lipid metabolism is also influenced by a number of adipocytokines including leptin, adiponectin, acylation-stimulating protein (ASP), tumour necrosis factor- α (TNF-α) and interleukin-6 (IL-6). Leptin, ASP and adiponectin all complement the actions of insulin since they increase muscle fatty acid oxidation and inhibit NEFA mobilization from adipocytes (Havel, 2002). Moreover, TNF-α and IL-6 increase IR via interference with the insulin signalling pathway (Chan *et al.,* 2005; Senn *et al.,* 2002). Although we did not measure these adipocytokines in the current study, it is possible that these physiological parameters may be more proximally related to apo-B48 concentration than anthropometric variables in overweight children. If so, accurate assessment of CVD risk in overweight children may also require the inclusion of adipocytokines.

5.3 Strengths and Limitations

5.3.1 Strengths: First, it was the first pediatric study to examine apo-B48 concentrations. Second, the SDS-PAGE, immunoblotting technique provided an accurate and precise method of measuring apo-B48. Third, the study provided a comprehensive assessment of CVD risk, particularly from a lipid profile perspective. Fourth, objective and comprehensive measures of body composition (e.g., MRI, DXA) were used. Fifth, the criteria used to define moderate and high risk was conservative and rigorous, consequently reducing the risk of overestimating those within the high risk category. Lastly, the study provided strong rationale to pursue further research in chylomicron metabolism as it relates to risk of CVD in children.

5.3.2 Limitations:

5.3.2.1 Normative pediatric data for apo-B48: There are limitations in comparing apo-B48 concentrations between children and adults, across different studies.

Although the values reported in this study were elevated compared to certain adult reports, it is possible that apo-B48 is simply elevated in children. Based on the increasing trend of other lipid parameters (TC, LDL-C, TG) with age (Jolliffe & Janssen, 2006; Jolliffe & Janssen, 2007), it is unlikely that apo-B48 concentration decreases with age. However, without reference data, one can only speculate on these trends. Further, the possibility of gender or ethnicity-based differences both in apo-B48 and other variables, supports the need to generate childhood reference data.

5.3.2.2 Sample size: Due to relative homogeneity in variable concentrations within the sample; it was statistically challenging to detect differences between groups. This limitation was particularly evident in the stepwise multivariate regression models; for every variable added to the model, the sample must increase by n = 10. Although both models included significant predictors both for apo-B48 concentration and risk group, a larger sample size would provide greater confidence in the exclusion / inclusion of variables. Pearson correlation analysis would be less influenced by outliers with a larger sample size and findings from our study would be more relevant to the general Canadian pre-pubertal overweight population.

5.3.2.3 Measures used to assess risk of CVD

Although we examined the main variables used to measure risk of CVD, there are a few other measures that would have created a comprehensive CVD risk profile. For example, Intimal medial thickness (IMT) is a common method of measuring atherosclerosis-induced vascular changes. Increased extracellular matrix secretion, collagenesis and smooth muscle hyperplasia cause intimal media thickening and compromised vessel dilation (McGill *et al.,* 1963). IMT tracks from childhood to adulthood and predicts cardiovascular events in adults (O'Leary *et al.,* 1999; Chambless *et al.,* 2000) and changes in IMT can be measured and used as an indicator of early atherosclerotic changes. Inclusion of IMT in our CVD risk assessment could have been used to examine the relationship between apo-B48 and early vascular changes.

It would have also been beneficial to include measurements of key adipocytokines in the study, particularly grehlin or adiponectin. As previously mentioned both are secreted from the adipocytes proportionally to the amount of visceral adipose tissue (Havel, 2002). Inclusion of either measure would have helped to examine the potential mechanistic links between abdominal fat deposition and chylomicron metabolism dysregulation.

5.4 Future Directions

A number of unanswered questions have been generated from this study and provide interesting avenues for future research. First, an ongoing study in our laboratory is evaluating apo-B48 concentrations in pre-pubertal, normal weight children. Reference data for apo-B48 will enable future studies to identify abnormal apo-B48 concentrations in pediatric groups. With reference data, retrospective analyses could be performed on these study data to determine whether apo-B48 concentrations were truly elevated. Second, future studies could include a larger sample size or focus on one gender exclusively. Third, for apo-B48 to have greater clinical importance in the pediatric population, a longitudinal analysis that includes fasting apo-B48 concentrations would be ideal. Fourth, establishing the link between apo-B48 in childhood and morbidity / mortality in adulthood would increase the impetus to study the intestinally-derived lipoprotein in children. Fifth, apo-B48 studies could examine the intestinal contribution to CVD risk and the influence of insulin resistance and excess weight on the intestine. Finally, more sophisticated kinetic studies, such as an oral fat test, would examine the post-prandial response to a fat challenge and determine the intestinal response and clearance mechanisms in children at risk of CVD.

Although adult studies have demonstrated that fasting apo-B48 predicts post-prandial lipemia, these findings have not been verified in children. It is possible that the secretion of chylomicrons remains tightly regulated in children, while the intestinal response following a lipid-containing meal is dysregulated; this is analogous to previous observations for fasting versus 2-hour glucose concentration. Use of the non-invasive breath test would be especially appropriate in the pediatric population, but would require validation against other methods of measuring apo-B48 such as the SDS-PAGE, immunoblotting technique used in this study.

5.5 Conclusion

In conclusion, apo-B48 is elevated in overweight pre-pubertal children in relation to normal weight and overweight adults in published studies. Although apo-B48 was strongly correlated with TG, it did not cluster with other established markers of CVD risk. While the initial conclusion may be that apo-B48 did not confer additional risk of CVD in the overweight, pre-pubertal children, the lack of difference between the moderate and high risk groups may suggest that elevated apo-B48 concentration exhibits a threshold phenomenon in children that relates to body fatness. It is possible that within this population of children, at increased risk of CVD due to their obesity, IR and dyslipidemia, apo-B48 is elevated and does not offer a unique contribution risk of CVD.

The most recent report from the NCEP Expert Panel (2002) stated "*some species of triglyceride rich lipoproteins, notably cholesterol-enriched remnant lipoproteins, promote atherosclerosis and predispose to coronary heart disease*," and that "*when TG levels are raised, LDL-C alone inadequately defines the risk associated with atherogenic lipoproteins*". Clearly, the importance of remnant lipoprotein measurement is beginning to be acknowledged in both the scientific and medical communities. Chylomicron research is at the forefront of increasing our understanding of atherogenesis and the contribution of the intestine to CVD risk. As prevalence of overweight, insulin resistant and dyslipidemia continues to increase in children, it will be important to understand the role of chylomicrons in early CVD risk.

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Appendix A: Pre-Screening form

PRE-SCREENING FORM

1) Initials: __________(First, Middle, Last) Gender: ☐Male ☐Female

2) Age: ___________ (years, months)

3) Date of Birth: ___/___/_____ (DD/MM/YYYY)

4) Does child appear to be: ☐Overweight ☐Average ☐Underweight ☐Unknown

Estimated:	Height	__________	(circle one: ft,in OR m)	☐Unknown
	Weight	__________	(circle one: kg OR lb)	☐Unknown
	BMI	__________	(kg/m^2)	☐Unknown
	BMI %ile	________		☐Unknown

5) Puberty:	Male:	Underarm Hair	☐No	☐Yes
		Pubic Hair	☐No	☐Yes
		Growth Spurt	☐No	☐Yes
	Female:	Breast Growth	☐No	☐Yes
		Vaginal Discharge	☐No	☐Yes

6) Is child free of any underlying medical conditions (see Visit 1, page 1-2)? Some conditions may be allowed – check with Dr. Mary Jetha if unsure. (add notes here)

____

7) Is child on any steroid medications? Must be off all oral steroids and high-dose inhaled steroids for ≥6 months. (add notes here)

____

Satisfies Pre-Screening Criteria (BMI ≥ 95th, Pre-pubertal, Free of medical conditions and steroid use) and is eligible for Visit 1:

☐Yes → Book Visit 1:
DATE: ______________________
TIME: ______________________
☐Confirmed by phone by ________

☐No → Would child/family like to be contacted regarding other research/programs in the future?

☐Yes ☐No

Appendix B: Adult Consent Form

UNIVERSITY OF ALBERTA

CONSENT FORM

Title of Study: **VISCERAL ADIPOSE TISSUE VOLUME, INSULIN RESISTANCE, AND ADIPONECTIN IN OVERWEIGHT PREPUBERTAL CHILDREN**

Principal Investigator: **Dr. Geoff Ball Ph: 407-3784**

Co-Investigators: **Dr. Mary Jetha (Study Coordinator) Ph: 407-8822, ask hospital operator to page**

Dr. Richard Lewanczuk	**Ph: 407-6277**	**Dr. Rhonda Rosychuk**	**Ph: 492-0318**
Dr. Linda McCargar	**Ph: 492-9287**	**Dr. Robert Couch**	**Ph: 407-6888**

(To be completed by the parent or guardian of the study participant):

	Yes	No
• Do you understand that your child has been asked to participate in a research study?	☐	☐
• Have you read and received a copy of the attached information sheet?	☐	☐
• Do you understand the benefits and risks involved in your child's participation?	☐	☐
• Have you and your child had an opportunity to ask questions and discuss this study?	☐	☐
• Do you understand that your child is free to withdraw from the study at any time. without having to give a reason and without affecting his/her future medical care?	☐	☐
• Has the issue of confidentiality been explained to you?	☐	☐
• Do you understand who will have access to your child's records, including personally identifiable health information?	☐	☐

- Do you want the investigator to inform your child's doctor that he/she is participating in this research study? If so, please give doctor's name: ______________________________
- Who explained this study to you?______________________________

I agree to my child's participation in this study: Yes ☐ **No** ☐
I agree to have my height and weight measured: Yes ☐ **No** ☐

____
Name of Child

Appendix C: Child Consent Form

Your autograph

If you want to do this study, you have to sign your name on this form. Your mom or dad will also sign a form agreeing that you may be in the study.

I agree to take part in this study: Yes ☐ No ☐

____
Signature of child (optional)

______________________________ ______________________________
Name of child Date

______________________________ ______________________________
Signature of witness Date

Appendix D: Magnetic Resonance Image Analysis Protocol

CHARM MRI Analysis Protocol

Step 1
On Paradigm computer
➢Open D:\ball
➢Select subject
➢Copy all haste_water_sat_subject files to jump drive

Step 2
➢Open Slice-o-matic
➢Drag and drop images into slice-o-matic to check anatomic position.
➢Change file names so images are numbered 1-60 starting from most inferior slice to most superior slice
ABC-039.MR.BALL_08.**01-20**.IMA (bottom slab)
ABC-039.MR.BALL_05.**21-40**.IMA (middle slab)
ABC-039.MR.BALL_11.**41-60**.IMA (top slab)

Step 3
➢Clear all images from Slice-o-matic
➢Modes>>Admin>>Select all>>Close Selection
➢File>>Quit
Now you are ready to analyse!

Step 4
➢Open Slice-o-Matic
➢Drag and drop bottom images into program

Step 5
➢Adjust image scale.
➢Click on + twice

Step 6
➢Adjust contrast simply by clicking once on the bar for white and once on the bar for black

Step 7
➢Change modalities
➢Modes>>Morpho

Step 8
➢Scroll up to image displaying top of femoral heads
➢Apply Mathematical Morphology 1

Step 9
➢Colour in VAT (red) and SAT (blue)
➢Pick paintbrush size and type and drag/click over sections of image
➢To erase, right click.

Step 10
➢Propagate you analysis up the subject's body.
➢Click "up" once.
➢Edit image (i.e., fill in missing sections, erase wrongly coloured areas)

- Repeat for each slab, saving intermittently.

Step 11

- Change modalities
- Modes>>Region Growing

Step 12

- Select thresholds by trial and error.
- Can usual set the lower limit of SAT to 130-160 and lower limit of VAT to 140-175.

Step 13

- Select colour and paintbrush size.
- Edit image (i.e., colour in missing spots and erase errors)
- Please see regions of ambiguity for further guidance.
- Remember to save!
- Double check that all .IMA images have been analysed and edited

Step 14

- Group the images so the three series are amalgamated into one.
- Modes>>Admin>>Select All>>Group Selection

Step 15

- Calculate VAT and SAT volume
- Tools>>TAG Surface/Volume>>DB Surfaces
- Save to desktop,

Step 16

- Open Slice-O-Matic Output
- Open "results" file using Excel
- Ensure all required slices appear on the table (should have 60)
- Save as subject-output

Step 17

- Edit Slice-O-Matic Output
- Refer to template

- Copy and paste appropriate columns from output (File name, slice thickness, TAG 1 (VAT) and TAG 3 (SAT) into template

Step 18

- Save file as subject [space] slice number included [-] results